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1 Glossary

ASC: Adult stem cell
Bmp2: Bone morphogenetic protein 2
CB: Cardiac body
CVPC: Cardiovascular progenitor cell
CMC: Cardiomyocyte
cTnT: Cardiac troponin T
EB: Embryoid body
ERK1/2: Extracellular signal related kinase 1/2
ESC: Embryonic stem cell
FBS: Fetal bovine serum
FGF4: Fibroblast growth factor 4
Gap1: GTPase activating enzyme 1
Grb2: Growth factor receptor binding protein 2
GSK3: Glycogen synthase kinase 3
Id 1: Inhibitor of DNA binding and differentiation 1
iPS: Induced pluripotent cells
JAK: Janus-Kinase,
Klf4: Krüppel-like factor 4
LIF: Leukemia inhibitory factor
LIFR: Leukemia inhibitory factor receptor
MAPK: Mitogen activated protein kinase
MEK 1/2: MAP-ERK kinase 1/2
mESC: Mouse embryonic stem cells
Nanog: Tir na og [celtic: Land of eternal youth]
Nkx2.5: NK2 transcription factor related, locus 5
Oct 3/4: Octamere binding factor 3/4
SDS-PAGE: Sodium dodecylsulfate polyacrylamide gel electrophoresis
PI3K: Phosphatidylinositol-3-OH kinase
pou5f: Pou-domain, class5, transcription factor1 (coding for Oct3/4)
qPCR: Quantitative polymerase chain reaction or real time PCR
Ras: Rat sarcoma
RT-PCR: Reverse transcriptase polymerase chain reaction
SF9: Insect cell line established from *Spodoptera frugiperda*
SCNT: Somatic cell nuclear transfer
Sos: Son of sevenless (D. melanogaster)
Sox2: SRY (sex determining region Y) box 2
SPARC: Secreted protein acidic and rich in cysteine
SRY: Sex determination region Y
STAT3: Signal Transducer and activator of transcription 3
Tbx3: T-box transcription factor 3
TGFβ: Transforming growth factor β
Wnt: Signaling pathway, the name Wnt is a combination of Wg (wingless) and Int gene

2 Introduction

Stem cells are one of the most important fields of research in modern molecular biology which will provide answers to fundamental scientific questions. Stem cells have the unique property of self-renewal and potency which means that they are able to differentiate into many different cell types. The regulatory mechanisms which regulate stem cell self-renewal and differentiation are still not fully understood. During embryogenesis embryonic stem cells (ESCs) differentiate into all cell types of the body in a strictly regulated manner. Thus, studying ESCs provides information on how they differentiate into the various cells of the body and how this differentiation is regulated. Furthermore, in organs of adult organisms like bone marrow, brain, skeletal muscle, skin, intestine liver, testis etc. stem cells are present called adult stem cells (ASCs). These ASCs have a restricted differentiation potential which means they can only differentiate into certain cells of an organ. ASCs have two essential functions which are tissue renewal and tissue damage repair. Continuous tissue renewal is necessary in organs like the skin or the intestine, where ASCs replenish aged cells. Tissue damage repair is needed for regeneration of injured organs. The regenerative function of ASCs in adults makes them especially interesting for medical researchers because of their potential use in stem cell therapy. ASCs, for example could be used for regeneration of injured tissues and organs or replacement of tissues. The first successful treatment using ASCs was the first successful hematopoietic stem cell transplantation, which was performed by Robert A. Good in 1968. Since then it is routinely used for the treatment of leukemia and related bone and blood cancers. Development of new adult stem cell therapies is still in progress.

A prime example for a potential application of a new stem cell therapy is cardiovascular disease (CVD). CVDs include several diseases of the heart and blood vessels like coronary heart disease, cerebrovascular disease, peripheral artery disease, rheumatic heart disease, congenital heart disease and heart failure. CVDs are the number one cause of death worldwide. In 2004 17.1 million people died from CVDs of which an estimated 7.2 million were due to coronary heart disease (WHO, 2009). During CVDs lack of oxygen in the heart tissue can lead to apoptosis of cardiomyocytes. This loss of cells is irreversible and often leads to a lethal progressive heart failure which can only be treated by heart transplantation. However, there are not enough donor organs for each patient and heart transplantation is a treatment but not a cure. The 1-year survival rate is over 80% and the average length of survival is 9.1 years (Jurt et al. 2002). For CVD-patients lack of donor organs could be circumvented and a complete healing could be achieved by transplantation of stem cells. Several clinical trials using bone-marrow derived hematopoietic stem cells and skeletal myoblasts were done in humans but showed modest success. Studies in animal models showed that stem cells are present in adult hearts (Joggerst and Hatzopoulos 2009). Studies

with rats showed that adult cardiac stem cells exist, which support myocardial regeneration (Beltrami et al. 2003). According to these studies, the most promising approach to heal and regenerate the injured heart tissue is to inject ASCs of the heart into the typical necrotic areas (Figure).

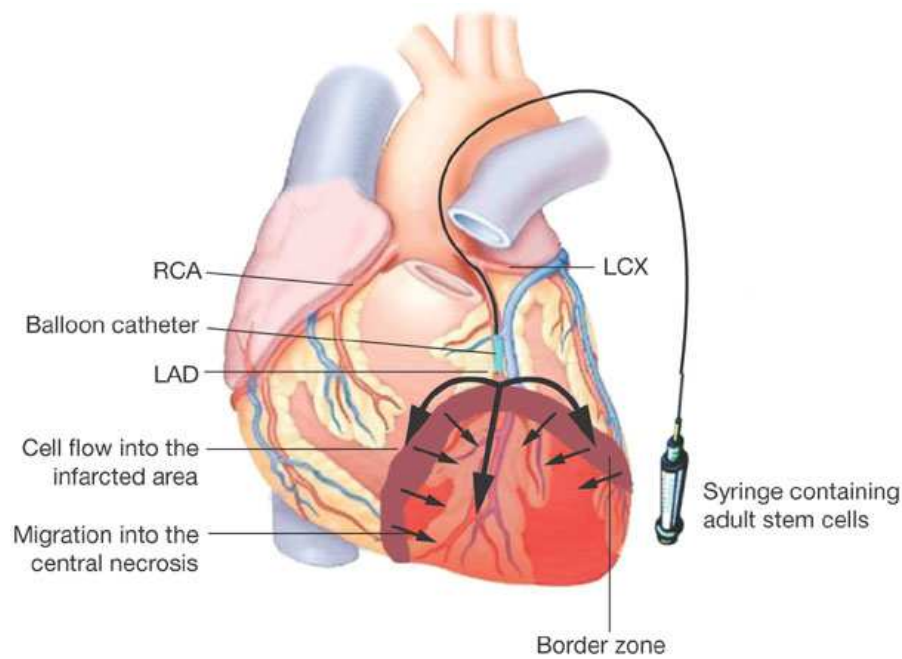


Figure 1: Illustration of a stem cell therapy for an infarcted heart. The balloon catheter enters the infarct-related artery and is placed above the border zone of the infarction. The catheter is then inflated and the cell suspension (including the patient's own cytokines) is infused at high pressure under stop-flow conditions. In this way, cells are transplanted into the infarcted zone through the infarct-related vessel system. Cells and cytokines infiltrate the infarcted zone. The arrows show the possible route of cell migration and cytokine infiltration. LAD, left anterior descending coronary artery; LCX, left circumflex artery; RCA, right coronary artery. (Adapted from Brehm 2006)

To obtain a sufficient quantity of adult cardiac stem cells for a stem cell therapy it will be necessary to cultivate and expand them in an in vitro culture. For directed and specific differentiation of ADCs to adult heart cells it is mandatory to understand which factors are responsible and which signalling pathways are regulated during differentiation. The first step is to establish an animal model for isolation and specific differentiation of cardiac ADCs. The ability to isolate, cultivate and handle cardiac stem cells will lead to the development of therapies for cardiovascular disease.

2.1 Stem cells

A stem cell is defined by two unique properties distinguishing it from any other cell:

- **Self renewal**, which means the ability to go through unlimited cell division cycles without differentiating.
- **Potency**, which means the capacity to differentiate into at least one cell type.

All stem cells have the capability of self renewal. The potency specifies the differentiation potential and differs in different stem cells. It can be subdivided into 5 classes:

1. **Totipotency**, is the ability to differentiate into any cell of the body. Totipotent cells can give rise to embryonic and extra-embryonic cells like placenta cells. A fertilized ovum is totipotent.
2. **Pluripotency**, means the ability to differentiate into any cell except extraembryonic cells. Examples are embryonic stem cells.
3. **Multipotency**, describes the ability of stem cells to differentiate into several different cell types. Hematopoietic stem cells for example give rise to all blood cells.
4. **Oligopotency**, is a property of stem cells which differentiate into few cell types. Lymphoid stem cells for example differentiate to B-, T- and natural killer cells.
5. **Unipotency**, is the ability to differentiate into only one cell type. An example is spermatogonial cells.

2.1.1 Sources of stem cells

- **Embryonic stem cells** are stem cells isolated from the inner cell mass of blastocysts and are pluripotent.
- **Somatic Cell Nuclear Transfer (SCNT)**. The nucleus of an oocyte is removed and the nucleus of a somatic cell is transferred into the oocyte. The resulting cell is pluripotent and gives rise to the embryo. All daughter cells are clones of the nucleus-donor. This process is also referred to as cloning. SCNT can be used for therapeutic cloning or reproductive cloning.
- **Induced pluripotent stem cells (iPS)** are genetically reprogrammed adult cells which resemble embryonic stem cells. Genetic modifications like expression of certain transcription factors are achieved by retroviral expression vectors. Possible differences to ESC are not fully tested yet. iPS cells are pluripotent.
- **Embryonic germ cells** can be isolated from the gonadal ridge of fetal tissue and are multipotent.
- **Adult stem cells** are stem cells which reside in tissues of the adult organism. ASCs have a limited potency and are multi-, oligo- or unipotent.

2.1.2 Embryonic stem cells

The fertilized oocyte is called zygote and represents the first ESC during embryogenesis. The zygote first undergoes cleavage in which it divides without growth. Afterwards, at the blastula stage the spherical embryo begins to form a fluid filled cavity called blastocoel. The resulting blastocyst contains cells inside the blastocoel called inner cell mass. These cells of the inner cell mass are the pluripotent ESCs which can be isolated for in vitro culture. Murine embryonic stem cells (mESCs) were first isolated from mouse blastocysts from the epiblast tissue of the inner cell mass by Martin Evans (Evans and Hunter 2002), Matthew Kaufman and separately and simultaneously by Gail R. Martin in 1981. In 1998 the first human embryonic stem cells were isolated by J. Thomson et al. (Thomson et al. 1998) (Figure 2).

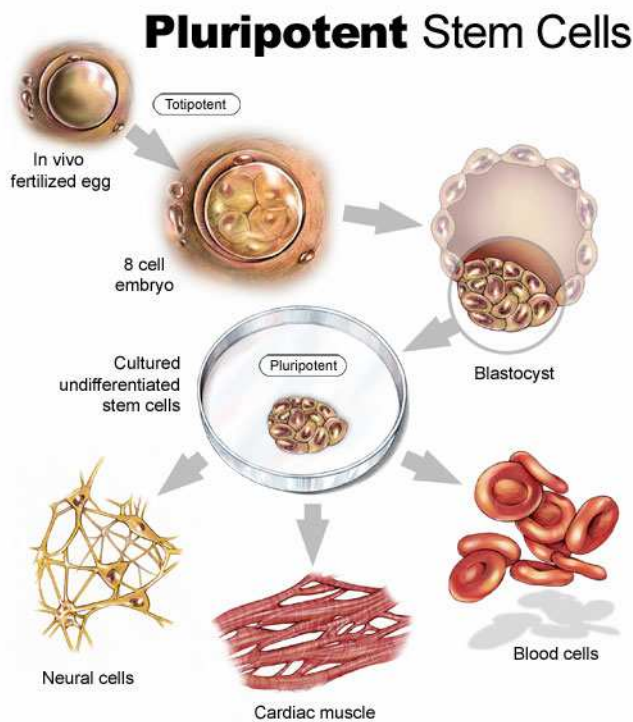


Figure 1: Embryonic stem cells (ESCs) isolated from the inner cell mass (ICM). ESCs can be isolated from the inner cell mass of an embryo at the blastocyst stage. ESCs from the inner cell mass are pluripotent and are able to differentiate to any cell type except extraembryonic tissue like the placenta. (Figure from Biotechnology Learning Hub)

Since pluripotent mESCs were first established they have been cultivated and maintained undifferentiated and pluripotent in vitro, using different combinations of feeder cells, cytokines, fetal calf sera and conditioned media to prevent differentiation. The most common procedure to cultivate isolated mESCs is to let them grow on a feeder cell layer consisting of STO SNL76/7 mouse fibroblasts. The feeder cells provide a matrix to which the ESCs can attach and they secrete cytokines like leukemia inhibitory factor (LIF). The secreted LIF together with bone morphogenetic protein 2 (BMP2), which is a component of the fetal bovine serum added to the medium, is sufficient to keep mESCs undifferentiated.

2.2 Embryoid bodies

Embryoid bodies (EBs) are aggregates of ESCs and represent an in vitro differentiation model of embryogenesis. A commonly used method to form EBs is to aggregate mESCs in hanging droplet culture. In hanging drops the ESCs form a compact sphere called embryoid body. After this aggregation ESCs start to differentiate into cell types of all three germ layers (endoderm, ectoderm and mesoderm). Every cell type present in the early embryo develops with the exception of trophoctodermal tissue which forms the placenta in vivo. Cysts form in the EBs and mesodermal cells differentiate to beating cardiomyocytes which can be observed within a few days of differentiation.

2.2.1 Regulation of murine embryonic stem cell pluripotency

In contrast to the classic view that mESCs have to be treated with factors to maintain pluripotency, Austin Smith et al (Ying et al. 2008) showed that mESCs have an innate capability to self-renew and replicate which doesn't depend on extrinsic factors. The auto-regulative expression of the three key transcription factors Oct3/4, Sox2 and Nanog (Masui et al. 2007) in mESCs is essential to maintain pluripotency. Therefore Oct3/4, Sox2 and Nanog are called stemness factors which are present in all stem cells.

- OCT3/4 (Octamere binding factor 3/4) is a homeodomain transcription factor and belongs to the POU (Pit/Oct/Unc) family. Oct3/4 is an essential stemness factor and all undifferentiated stem cells express Oct3/4. In mESCs Oct3/4 expression prevents differentiation. A knockdown of Oct3/4 induces differentiation (Zaehres et al. 2005). If Oct3/4 is repressed at the morula stage of the mouse embryo, mESCs differentiate to trophoctoderm (Figure3). Oct3/4 expression is also observed in several adult stem cells (Ling et al. 2006).
- Nanog is a NK-2 class homeobox transcription factor which is also essential for self-renewal of mESCs. mESCs start to differentiate to primitive endoderm without Nanog expression (Mitsui et al. 2003). Overexpression of Nanog in mESCs is sufficient to keep them undifferentiated in absence of LIF.
- Sox2 (sex determining region Y)-box 2 is a transcription factor and belongs to the SRY related HMG-box (SOX) transcription factor family. Sox2 expression is essential for mESC self renewal.

Oct3/4, Sox2 and Nanog build a transcriptional network and regulate the expression of each other by feedback loops. Oct3/4 and Sox2 bind to each other and activate Oct3-Sox enhancers which regulate stem cell specific gene expression and the expression of the 3 stemness factors themselves (Masui et al. 2007). How the regulation exactly works is not fully discovered yet. Downregulation of the three stemness factors correlates with differentiation.

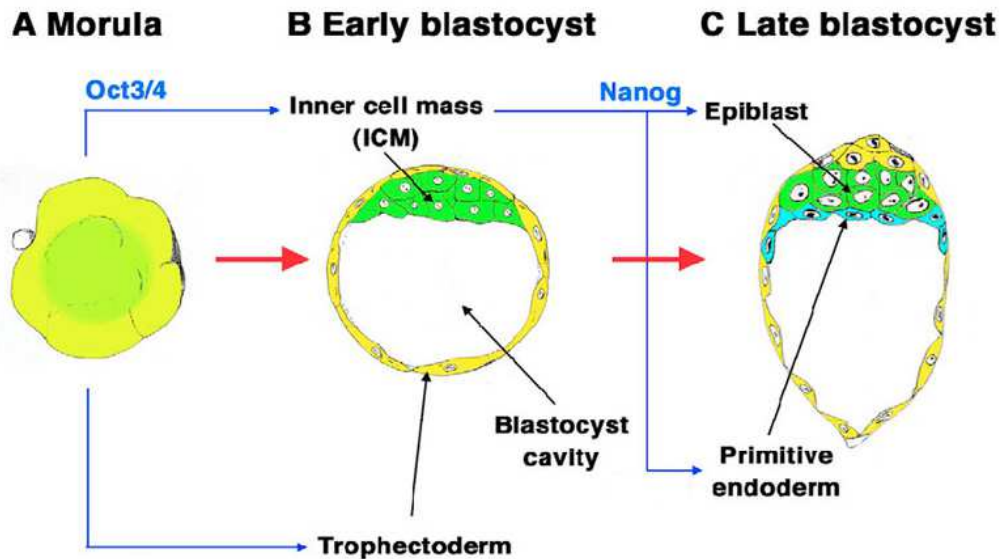


Figure 2: Regulation of stemness factors Oct3/4 and Nanog during embryogenesis. At the morula stage cells without Oct3/4 expression differentiate to trophoblast. In the early blastocyst cells of the inner cell mass can only differentiate to pluripotent epiblast cells if Nanog is expressed, otherwise they become primitive endoderm. The epiblast tissue is the source of pluripotent ESCs which express Oct3/4, Nanog and Sox2. (Adapted from Niwa, 2007)

mESCs are susceptible to differentiation signals, which may drive them towards commitment when expression and autocrine secretion of fibroblast growth factor 4 (FGF4) is induced by Oct3/4 and Sox2. Upon activation of the FGF4-receptor by FGF4 the tyrosine kinase of the FGF4 receptor activates the MAPK (mitogen activated protein kinase) Erk1/2 (Extracellular Signal-Regulated Kinases 1 and 2) pathway which ends the pluripotent state and leads towards commitment (Ying et al. 2008).

The susceptibility to commitment can be prevented by specifically inhibiting the Erk1/2 pathway activated by FGF4 (Figure 4). The 2 molecules SU5402 and PD184352 specifically inhibit the FGF4 receptor tyrosine kinase thereby blocking the activation of the ERK1/2 kinase. Inhibiting the ERK1/2 pathway is sufficient to maintain pluripotency in mESCs but the apoptosis rate is high and cell growth and viability is impaired. To overcome this problem a new specific inhibitor of glycogen synthase kinase 3 (GSK3) the chemical CHIR99021 was utilized, referring to reports (Sato et al. 2004) that inhibition of GSK3 enhances mESCs viability. mESCs treated with CHIR99021 in combination with SU5402 and PD184352 remained pluripotent, viable and showed a normal proliferation rate. In summary, it was shown that commitment can be inhibited by blocking the ERK1/2 pathway and by GSK3 inhibition when mESCs are treated with the 3 specific inhibitors CHIR99021, SU5402 and PD184352, called '3i'.

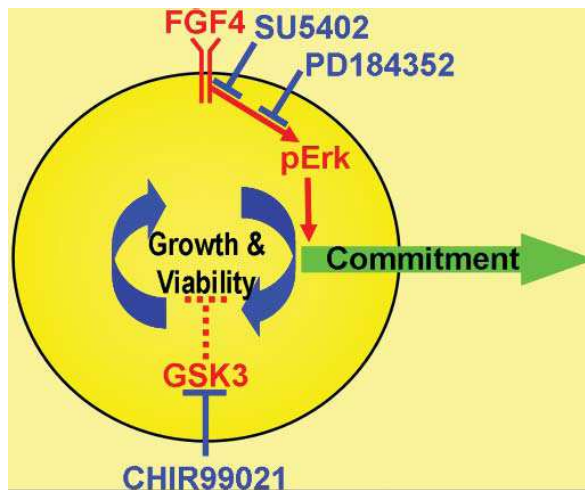


Figure 4: Inhibition of commitment in mESCs by blocking the Erk1/2 MAPK-pathway and GSK3. FGF4 induced commitment can be inhibited upstream of the ERK1/2 pathway by inhibitors SU5402 and PD184352. Additional inhibition of GSK3 by CHIR99021 is needed to maintain cellular growth and viability (Adapted from Ying et al, 2008).

In conventional mESCs culture commitment was always blocked with LIF and BMP2, but the exact pathways responsible were unknown. Although LIF and BMP2 act downstream of the MAPK pathway, administering LIF and BMP together is sufficient to maintain mESCs undifferentiated (Figure 2). mESCs can be successfully propagated by cultivation on a feeder cell layer of SNL76/7 STO mouse fibroblasts which secrete LIF. Alternatively LIF can be administered to the cultivation medium. Furthermore the fetal bovine serum or fetal calve serum in the medium with which cells are fed contains BMPs.

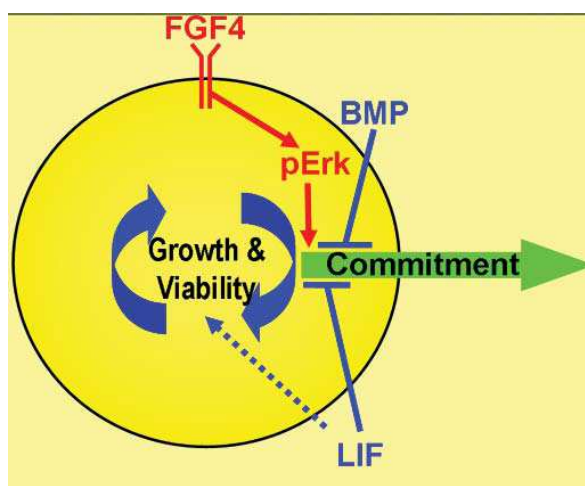


Figure 5: BMP2 and LIF inhibit commitment downstream of ERK1/2 pathway. BMP2 present in the serum of cell culture medium and LIF secreted by feeders are sufficient to inhibit FGF4 induced commitment in mESCs. Viability is increased by LIF(Adapted from Ying et al, 2008).

2.3 Leukemia inhibitory factor and bone morphogenetic protein 2 in self-renewal

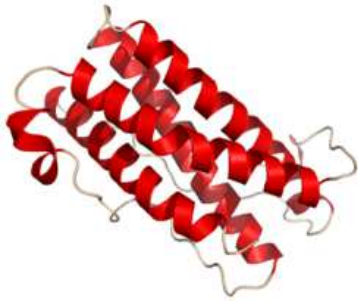


Figure 6: Structure of LIF (RCSB Proteine Data Bank)

Leukemia inhibitory factor (LIF) (Figure 6) derives its name from the ability to induce terminal differentiation of myeloid leukemic cells. It is a glycoprotein belonging to the Interleukin-6 class cytokines. LIF is pleiotrop, it has different effects on different cell types and tissues. On the one hand it is used to maintain mESCs undifferentiated in cell culture, on the other hand it is known for the growth promotion and cell differentiation of different types of target cells, and its influence on bone metabolism, cachexia, neural development, inflammation and embryogenesis. In mESCs culture LIF is added to the medium or secreted by feeder cells because mESCs spontaneously differentiate without LIF. In the developing embryo LIF is expressed in the trophoectoderm, and the LIF-receptor (LIFR) is expressed throughout the inner cell mass (ICM). As mESCs are derived from the ICM at the blastocyst stage, removing them from the ICM also removes their source of LIF. Without LIF mESCs lose their totipotency and start to differentiate. Therefore LIF has to be added to the medium in mESCs culture. mESCs are usually cultivated on a feeder cell layer, consisting of transformed mouse fibroblasts which express and provide them with LIF. Interestingly LIF is not required for culture of human embryonic stem cells.

The mouse LIF-gene codes for three different isoforms of LIF: truncated LIF (T-LIF), matrix associated LIF (M-LIF) and diffusible LIF (D-LIF), which are generated by alternative splicing (Rathjen et al. 1990). M and D-LIF have the same size and sequence except in their N-terminal aminoacid-sequence, a putative leader sequence, and are both secreted by cells. Truncated LIF remains inside the cell. Although M-LIF and D- LIF are both secreted they may have different effects (Stary et al. 2006).

In mESCs LIF binds to heterodimers of gp130 receptor and LIF receptor (LIFR) which leads to activation of 3 different pathways (Figure 7): The Jak-Stat3 pathway, phosphatidylinositol-3-OH kinase (PI3K)-Akt and Erk1/2 pathway (Niwa et al. 2009).

1. Notably the Jak-Stat3 pathway is the only one of the 3 LIF-activated pathways in mESCs which is activated by LIF alone. After heterodimerization of gp130 and LIFR associated Janus kinase (Jak) becomes activated by phosphorylation and phosphorylates signal transducer and activator of transcription 3 (Stat3). Phosphorylated Stat3 homodimers translocate into the nucleus and activate the nuclear transcription factor Krüppel-like factor 4 (Klf4) which induces Sox2 and Nanog transcription.
2. The PI3K pathway is regulated by LIF, insulin, insulin-like growth factors and a rat sarcoma (Ras) like protein kinase called Eras (Takahashi et al. 2003), which is constitutively active in mESCs. Activated PI3K leads to activation of T-box transcription factor 3 (Tbx3) and Nanog (Storm et al. 2007).
3. The Erk1/2 pathway is a pathway which promotes differentiation in mESCs and is also induced by the autocrine FGF4 signaling (see chapter 4.1). Erk1/2 pathway is activated when Jak phosphorylates “Growth factor receptor-bound protein 2” (Grb2) which starts the MAPK signaling cascade including the proteins “Son of Sevenless” (Sos), Ras, Raf and Erk1/2. Erk1/2 promotes differentiation by stimulating the nuclear export of Tbx3.

The Jak-Stat3 pathway which is solely regulated by LIF induces self renewal in mESCs. The Erk1/2 pathway induces differentiation in mESCs. The PI3K and the Erk1/2 pathways are influenced by other signals than LIF as well. Interestingly all 3 pathways act on Klf4 and Tbx3 which influence the expression of the 3 stemness factors Oct3/4, Sox2 and Nanog. Thus, these two transcription factors link LIF to the transcriptional network of self renewal. Recent experiments confirmed this finding when mESCs were transfected with Tbx3 and Klf4. Transfected mESCs could be cultivated and remained pluripotent in absence of LIF (Niwa et al. 2009).

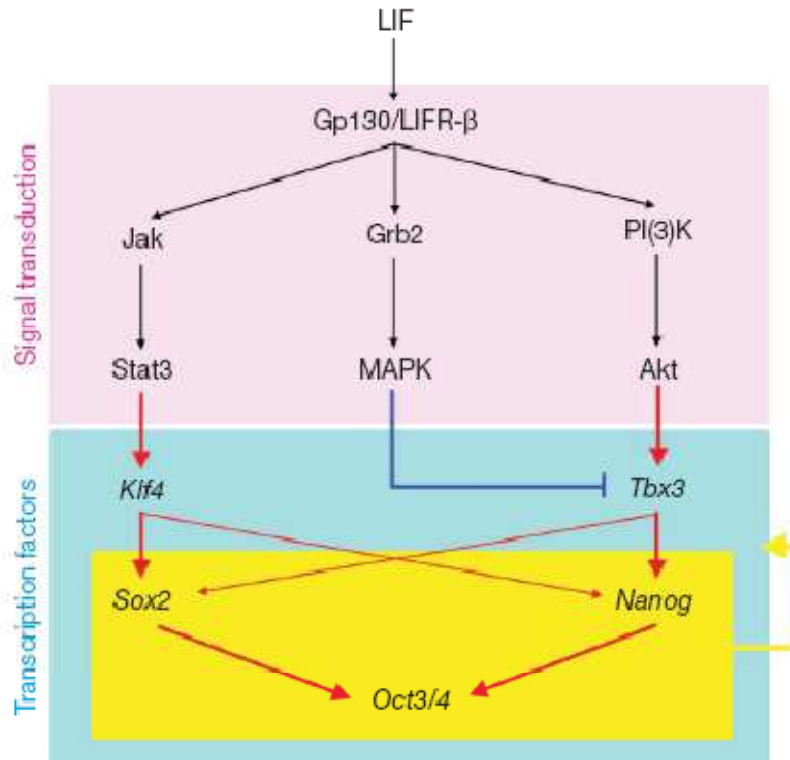


Figure 7: LIF is connected to the pluripotency network: LIF induces Jak/STAT3 and PI3K (Akt) pathways which activate transcription factors Klf4 and Tbx3. Activated Klf4 and Tbx3 upregulate Sox2 and Nanog expression. The alternatively activated MAPK pathway inhibits pluripotency by inhibiting nuclear localization of Tbx3 (From Niwa, 2009).

Nevertheless, previous experiments with EBs showed a promoting effect of LIF on cardiomyogenesis in studies by Bader et al (Bader et al. 2001). Moreover, LIF activates Stat3 by phosphorylation and in the cardiac marker gene *Nkx2.5* Stat3-specific binding sequences are present in the promoter region.

2.4 Bone morphogenetic proteins

The cytokine bone morphogenetic factor 2 (BMP2) belongs to the transforming growth factor β (TGF- β) super-family. The BMP group comprises at least 7 BMPs. Its name is derived from the ability to induce ectopic bone formation in rodents. BMP2 binds to heterodimers of BMP receptor type I (BMPRI) and BMP receptor type II (BMPRII). The serine threonine kinase domain of activated BMPRI and BMPRII phosphorylates Smad1/5/8 which binds to Smad4 and translocates to the nucleus. In the nucleus the Smad complex can inhibit or promote gene expression when binding to transcriptional activators or transcriptional repressors. In mESCs culture BMPs are present in the fetal calf serum. When a serum free medium and no feeder cells are used, a combination of LIF and BMP4 or BMP2 is sufficient to maintain pluripotency and self renewal (Ying et al. 2003). In undifferentiated mESCs BMP activates Smad1/5/8 which promotes

inhibitor of differentiation (Id) expression. mESCs transfected with Id remain pluripotent when cultured in serum free medium with LIF.

BMP2 expression is essential during embryogenesis. Without BMP2 early embryos have an abnormal cardiac development and fail in amnion and chorion formation (Zhang and Bradley 1996). BMP2 is essential for early heart development and induces expression of cardiac marker genes like Nkx2.5 and GATA4 (Schlange et al. 2000).

Besides maintaining pluripotency in mESCs, BMPs like LIF can also induce differentiation. In neural progenitors, differentiation to astrocytes is induced by LIF and BMP2. The transcriptional coactivator p300 binds to both LIF induced STAT3 and BMP induced SMAD1 (Nakashima et al. 1999). Thus, LIF and BMP can cooperatively induce differentiation in neural progenitor cells or maintain pluripotency in mESCs.

2.5 Secreted protein acidic and rich in cysteine (SPARC, Osteonectin, BM-40)

SPARC, also called osteonectin, 43K protein or BM-40, is an extracellular Ca^{2+} binding matricellular glycoprotein and has versatile effects on cells undergoing migration, morphogenesis and differentiation. Its main functions are counter-adhesion and anti-proliferation which are induced by activating different pathways. SPARC consists of 3 domains. The N-terminal acidic Ca^{2+} -binding domain binds Ca^{2+} with low affinity (Maurer et al. 1992) and interacts with hydroxyapatite (Romberg et al. 1985). It has therefore been implicated in the mineralization of cartilage and bone (Figure 8). The second domain contains follistatin-like sequences and stimulates cell proliferation and angiogenesis (Funk and Sage 1993). The third domain is extracellular and consists of a largely α -helical high affinity Ca^{2+} -binding domain with a binding site for collagen IV. SPARC is expressed in different tissues and has an effect on remodeling and repair. During embryogenesis SPARC is expressed in heart primordia, somites, and extraembryonic membranes. SPARC interacts with growth factors like VEGF, FGF2 and PDGF and binds to extracellular matrix and cell surface proteins. It was shown that SPARC is expressed during muscle development and during regeneration of muscle tissue. Expression of SPARC was found in myoblasts and also in myotubes and muscle fibers. The degree of SPARC expression in regenerating myotubes correlates with the severity of myotube injuries (Jorgensen et al. 2009).

SPARC activates the TGF- β pathway and interacts with integrin linked kinase 1 (ILK1) (Brekken and Sage 2000). ILK1 phosphorylates protein kinase B (PKB/Akt) and GSK3 (Persad et al., 2001b). Phosphorylation of GSK3 leads to upregulation of the Wnt-pathway and nuclear β -catenin. Wnt signaling upregulates the expression of the

cardiac transcription factor Nkx2.5. In myogenic differentiation ILK1 also regulates the Erk1/2 pathway by preventing Erk inactivation during myogenic differentiation.

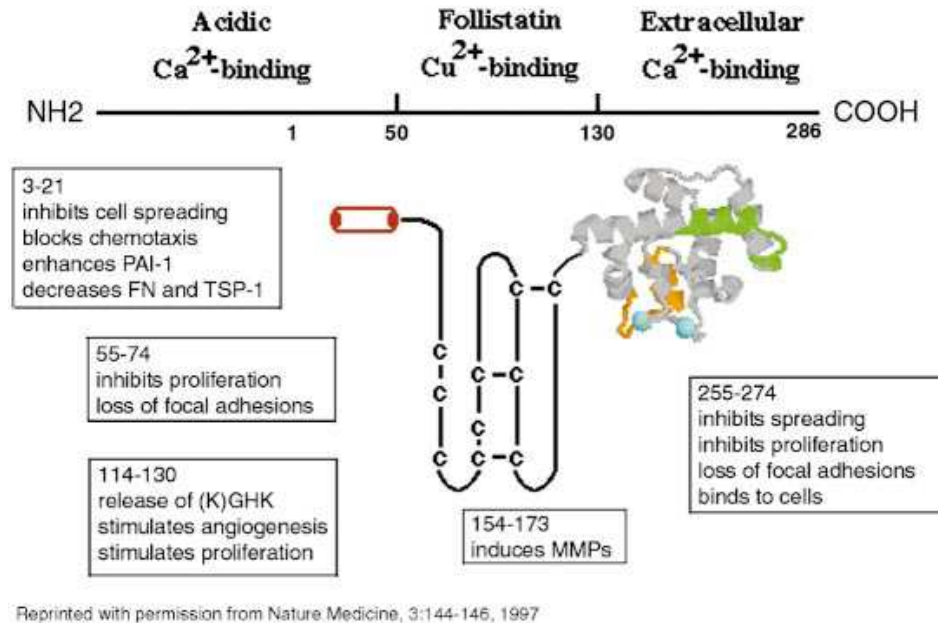


Figure 8: Structure of SPARC: SPARC consists of 3 domains, an acidic Ca^{2+} -binding domain, a follistatin-like domain, and a Ca^{2+} -binding extracellular domain (Adapted from Qi Yan and E. Helene Sage, 1999).

Martina Stary showed that SPARC is secreted by parietal endoderm and promotes cardiomyogenesis in EBs. Inhibition of SPARC inhibits cardiomyogenesis in EBs. In conjunction with BMP2, SPARC activates transcription of the cardiac marker gene Nkx2.5 (Stary et al. 2005). Thus, SPARC and BMP2 may enhance cardiomyogenesis in cardiovascular progenitor cells as well.

2.6 Cardiovascular progenitor cells (CVPCs)

Georg Weitzers laboratory has established 11 clones of a putative cardiovascular progenitor cell line (CVPC) isolated from heart tissue of neonatal mice. Heart cells from 1 to 2 day old 129SvxC57BL6J hybrid mice, with a neomycin resistance gene insertion in one allele of the *hdac1* locus, were isolated and co-cultured with an equal number of AB2.2 mouse embryonic stem cells on mitotically inactivated SNL76/7 mouse fibroblasts and LIF-expressing parietal endoderm cells. After splitting 1:3 every third day for 10 passages, the cells were selected for neomycin resistance by treatment with G418, removing all AB2.2 mESCs (Pasteiner 2003; 2006). To exclude contamination by mESCs, 100 cells obtained from one colony were seeded onto a 6cm plate on LIF-expressing SNL76/7 feeder cells and treated with G418 for another 10 days. 12 colonies were picked and 11 clonal cell lines could be derived, expanded and frozen (Wiedner 2008). The morphology of CVPCs is different to mESCs and karyotyping of those cells showed that fusion products of mESCs and heart cells can be excluded

(Wiedner 2008, Höbaus 2009). CVPCs can be maintained undifferentiated by cultivation on SNL76/7 feeder layers (Wiedner 2008). The CVPC cell line is still under investigation and self-renewal capacity and differentiation-potency is being determined.

2.7 Conclusions and experimental setup

To characterize CVPCs, first of all adult stem cell or progenitor cell characteristics had to be demonstrated. Based on research papers on CVPCs and mESCs an experimental strategy was developed for my diploma thesis. Wiedner (Wiedner 2008) showed that the CVPCs can be maintained undifferentiated just like mESCs by cultivation on LIF expressing feeder cells and feeding fetal calf serum containing BMPs. Therefore CVPCs will start differentiating in absence of feeder cells and without LIF. If CVPCs are multipotent but not pluripotent they will have a specific and restricted cardiovascular differentiation potential. Experiments in hanging drop culture and monolayer differentiation experiments should prove this. If CVPCs are stem cells, stem cell marker gene expression like Oct3/4 and Nanog and telomerase activity should be present. Expression of cardiovascular marker genes should be measured in undifferentiated and differentiated CVPCs. On protein level cardiovascular marker proteins like cardiac troponin T should be detectable by immunofluorescence in differentiated CVPCs.

After successful maintenance of CVPCs and after verifying characteristics of a cardiovascular progenitor cell, differentiation mechanisms have to be investigated. In mESCs LIF and BMP2 inhibit differentiation and therefore have to be removed for differentiation. However, in somatic cells both factors induce differentiation. Therefore, a dual role of LIF and BMP2 in CVPCs is possible. LIF and BMP may activate different pathways to maintain self-renewal or pathways to induce differentiation (see chapter 4.2 and 4.3) depending on co-factors, dosage, cell density and time interval of treatment.

SPARC promotes cardiomyogenesis in vitro in EBs and upregulates cardiac marker gene Nkx2.5 expression. Furthermore, SPARC is expressed in fetal myotubes and in regenerating myotubes. Thus, it is probable that SPARC promotes cardiomyocyte differentiation and promotes cardiomyocyte specific differentiation in CVPCs as well. SPARC is connected to the Wnt-pathway via ILK1 and Akt/PKB dependent phosphorylation and Wnt upregulates Nkx2.5 (Liu et al. 2009). Proof of this new pathway would explain upregulation of Nkx2.5 by SPARC.

3 Materials and Methods

3.1 Chemicals for molecular biology

Acetic acid	Merck, D
Acrylamide	BioRad, USA
Agarose, Biozyme LE	Biozyme, D
Aprotinin	Sigma, USA
BCIP (5'Brom-4'Chlor-3'Indoylphosphat)	Diagnostic Chemicals Limited, D
β-Mercaptoethanol	Loba Feinchemie, A
Bromphenolblue	Sigma, USA
BSA	Roth, D
Coomassie Brilliant Blue R250	Merck, D
DAPI	Sigma, USA
Dabco	Sigma, USA
dNTPs	MBI Fermentas, Lithuania
Dimethylsulfoxid (DMSO)	Acros, B
Dimethylformamid	Fluka, CH
Dithiothreitol (DTT)	Acros, B
EDTA	Acros, B
Ethanol	Merck, D
Ethidiumbromid	Fluka, CH
Formaldehyde	Merck, D
Glycerin	Merck, D
Hydrochloric acid (HCl)	Acros, B
Imidazole	Sigma, USA

Leupeptin	Sigma, USA
Methanol	Merck, D
MgCl ₂	MBI Fermentas, Lithuania
Mowiol 2-88	Hoechst, D
NEB Buffer	New England Biolabs, USA
Nitrotetrazolium Blue Chloride (NBT)	Fluka, CH
Ni-NTA agarose beads	Quiagen, D
PCR buffer without MgCl ₂	MBI, Fermentas, Lithuania
Pepstatin A	Sigma, USA
Ponceau-S	Sigma, USA
Reverse transcriptase buffer	Invitrogen, USA
SDS	BioRad, USA
Sodiumbicarbonate	Sigma, USA
Sodiumchloride	Salinen Austria, A
Sodiumhydrogencarbonate	LifeTechnologies, USA
Sodiumhydrogenphosphate	Roth, D
Sodiumhydroxide	Merck, D
Sodiumthiosulfate	Merck, D
Trichloracetic acid	Merck D
Tris Base	LifeTechnologies, USA
Tween-20	Sigma, USA

3.2 Chemicals for cell culture

β-Mercaptoethanol	Loba, A
Amphotericin B	Invitrogen, USA
D-Glucose	Acros, B

DMEM powder	LifeTechnologies, USA
DMSO (Dimethylsulfoxide)	Sigma, USA
Fetal Bovine Serum (FBS)	HyClone, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Fetal Bovine Serum (FBS)	Sigma, USA
Gelatine	Difco, USA
Glycine	Applichem, D
L-(+)-Glutamine	Acros, B
Mitomycin C	Acros, B
Penicillin G Kaliumsalz	Merck, D
Penicillin / Streptomycin (P/S)	GibcoBRL
Potassium chloride	Sigma, USA
Recombinant M-LIF	Chemicon International
Sf-900 SFM medium	GibcoBRL
Sodiumhydrongencarbonate	LifeTechnologies, USA
Streptomycin	Sigma, USA

3.3 Enzymes

DNase I, RNase free	Fermentas
RevertAid TM M-MuLV RT	Fermentas
RiboLock RNase Inhibitor	Fermentas
RNaseOUT	LifeTechnologies, USA
Taq DNA Polymerase	MBI Fermentas, Lithuania
Trypsin	LifeTechnologies, USA

3.4 Cell lines

SNL76/7-Fibroblasts:

This mouse fibroblast strain was established by Allan Bradley. It is based on STO fibroblasts, which were stably transfected with a neomycin resistance expression vector and a LIF expression vector (McMahon and Bradley, 1990).

Ab2.2. Embryonic stem cells

The embryonic stem cell line Ab2.2 was isolated by Allan Bradley from the inner cell mass of blastocysts of 129Sv mice in 1991.

Cardiovascular Progenitor Cells:

CVPC cell lines were isolated by Wolfgang Weber from heart tissue of HDAC1 +/- mice. This mouse line encodes a neomycin-resistance gene and can therefore survive selection with neomycin or neomycin analogs (e.g. G418). In order to increase survival rate of successful isolated cells, heart tissue derived cells were co-cultured with murine wild type embryonic stem cells Ab2.2. This co-culture was used to supply possible cardiovascular progenitor cells with external factors that are usually supplied by the stem cell niche. Since niche conditions are unknown, the presence of embryonic stem cells and the proteins, cytokines, etc. secreted by these, was thought to substitute for the stem cell niche. After 10 passages of co-culture (3T3 protocol) on feeder cells a selection against hypertrophic cells and towards fast replicating cells had already taken place. Addition of the neomycin analog G418 resulted in death of Ab2.2 embryonic stem cells. CVPC cells were cloned by seeding one cell per plate and expanding this culture under treatment of G418. 12 cell lines were derived named A3, A5, B3, B5, C3, D3, D5, E3, F3, G3, and H3.

SF9-cells:

SF-9 is an immortalized cell line derived from pupal ovarian cells of *Spodoptera frugiperad*. The SF-9 cell line is highly susceptible to infections with baculoviruses and can be used with all baculovirus expression vectors. SF9 cells are commonly used to produce recombinant proteins.

3.5 Culture of mammalian cells

3.5.1 Washing of glass pipettes:

After usage the cotton plug is removed and pipettes are put into vessels containing a mixture of water and hypochlorite. Glass pipettes are removed from the vessels and rinsed with fresh tap water for at least four hours. Afterwards pipettes are moved to a vessel containing Milli-Q water and are left there overnight. Pipettes are dried at 80°C for 4 hours and then placed in pipette boxes and baked at 180°C for 8 to 10 hours. If pipettes are contaminated with soap they can no longer be used for tissue culture of embryonic stem cells.

3.5.2 Washing of bottles and other cell culture glass ware

A small amount of hypochlorite is added to bottles and filled up to the top with tap water. After 15-30 minutes bottles are rinsed at least five times with tap water and then filled up with Milli-Q and left overnight. Then the bottles can be emptied and left upside-down till dry. Bottles are closed halfway and autoclaved at 120°C at 1.4 bar for 20-30 minutes. Magnets and other tissue culture appliances are also washed with hypochlorite and Milli-Q prior to use and should solely be used for tissue culture and never come in contact with soap.

3.5.3 Media and solutions for mammalian cell culture

10x PBS (Phosphate buffered saline)

NaCl	80 g
KCl	2 g
Na ₂ HPO ₄ ·7H ₂ O	10.72 g
KH ₂ PO ₄	2 g

Dissolve salts in 800 ml MilliQ water and titrate to a pH-value of 7.2 with a saturated Na₂HPO₄·7H₂O-solution. Fill to 1 l with MilliQ water. Sterile filtration (Nalgene Filter, Nalgene Membrane, 0.22 µm pore width). Store the stock solution at room temperature in the cell culture room.

1x PBS

Autoclave 450 ml MilliQ water in a 500 ml cell culture bottle. Fill the bottle to 500 ml with 50 ml 10xPBS in the hood.

100x GPS (Glutamine-Penicillin-Streptomycin)

NaCl	80 g
Penicillin	1.5 g
Streptomycin	2.5 g
L-(+)-Glutamine	14.6 g

Dissolve substances in 500 ml MilliQ water, sterile filtration, aliquot 40 ml in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

100x β -Mercaptoethanol

1xPBS	200 ml
β -Mercaptoethanol	144 μ l

Mix solution, sterile filtration and aliquot in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

Trypsin

NaCl	3.5 g
D-Glucose	0.5 g
Na ₂ HPO ₄ ·7H ₂ O	0.09 g
KCl	0.185 g
KH ₂ PO ₄	0.12 g
EDTA	0.2 g
Trypsin	1.25 g
Tris Base	1.5 g

Dissolve the chemicals in MilliQ water, titrate to pH 7.6 with concentrated HCl, fill to 500 ml with MilliQ water and sterilize by filtration and aliquot 40 ml in 50 ml Falcons. Store at -20°C in the cell culture refrigerator, store at 4°C after thawing.

1% gelatine stock solution

Solve 100 g gelatine (Difco) in 1 l MilliQ water. Sterilize by filtration. Store at room temperature in cell culture.

0.1% gelatine solution

Autoclave 450 ml of Milli-Q water in a cell culture bottle and add 50 ml of the 1% stock solution in the hood and mix.

Dulbeccos modified eagles medium (DMEM)

To prepare DMEM a 5 l Erlenmeyer is filled with 4.5 l Milli-Q water. Exactly one half of a 10 l DMEM package (Gibco) is added under stirring. Upon dissolution of the powder 18.5 g of sodium bicarbonate is added. The Erlenmeyer is filled to 5 l and the medium is filtered through sterile filters into autoclaved bottles. 4 ml of medium per bottle is left aside and incubated at 37°C over night. The medium is analyzed for contaminations under the microscope the next day.

3.6 Nutrition media for cell culture

M10Gi: for fibroblasts:

DMEM

10% Fetal bovine serum from Gibco

1x GPS

M15Hi: for ESC and CVPC:

15% = 30 ml Fetal bovine serum from HyClone

1% = 2 ml GPS

1% = 2 ml β -Mercaptoethanol

To 200 ml with DMEM

M15Si: for CBs:

15% = 60 ml Fetal bovine serum from Sigma

1% = 4 ml GPS

1% = 4 ml β -Mercaptoethanol

To 400 ml with DMEM

Freezing medium:

60% DMEM

20% FBS (Gibco for fibroblasts, HyClone for ES cells)

20% DMSO

Note: Always add DMEM first; when mixed use it immediately afterwards

3.7 Media and solutions for insect cell culture

SF9 thawing and adaptation medium:

Same as culture medium, only with 20-5% FCS

SF9 culture medium:

Sf-900 medium

2% FBS

1% P/S

Infection medium:

Sf-900 medium

2% FBS

SF9 freezing medium:

20% FBS

20% DMSO

60% Sf-900 medium

Baculovirus freezing medium:

Add 4% FBS to CM containing viral particles

3.7.1 Coating of cell culture plates

Cell culture plates have to be gelled to serve as a matrix for the cells to adhere. Apply at least 2 ml of 0.1% gelatine solution per 6-well and 6 cm plate, or 3 ml per 10 cm. Leave gelatine solution on plates for two hours at room temperature, then suck off the solution completely with a Pasteur pipette. Use the cell culture plates directly after gelling.

3.7.2 Maintenance of SNL67/7

SNL67/7 fibroblast feeder cells express LIF (Leukemia inhibitory factor).

3.7.3 Thawing of SNL76/7

10 cm culture plates must be coated with gelatine before starting the defrosting procedure. SNL67/7 are stored in cryotubes at -180°C in liquid nitrogen. Cryotubes are carefully removed from liquid nitrogen tank and defrosted in a 37° bath until a thin icicle is left in the tube. Cryotubes are disinfected with 70% EtOH, dried, placed in the laminar flow and flamed before and after opening. The content (1.8 ml) is transferred into a 15 ml falcon tube and 10 ml M10Gi are added drop by drop while mixing over a time period of at least 5 minutes to prevent an osmotic shock. 15 ml Falcons are centrifuged at 1000 rpm for 7 minutes at room temperature (Heraeus, Biofuge). After the supernatant is discarded pellets are resuspended in 1 ml of M10Gi. Another 3 ml of M10Gi is added to 15 ml Falcons and then transferred onto a gelatin coated 10 cm culture dish. Falcons are washed with 4 ml of fresh M10Gi and the solution is added to the culture dish.

3.7.4 Cultivation of SNL76/7

SNL67/7 cells are cultured at 37°C and 5% CO_2 . Medium must be replaced by fresh M10Gi if color switches to orange or yellow. Cells must be split when plate is confluent.

3.7.5 Splitting of SNL76/7

When confluent, cells must be split onto new gelatin coated culture dishes. Medium is discarded and plates are washed with 1xPBS. PBS is applied to remove leftover of M10Gi that would inhibit function of trypsin. After PBS is sucked off 1 ml of trypsin is evenly applied to the culture plate and plates are incubated at 37°C and 5% CO_2 for 5 minutes. 3 ml of fresh M10Gi are added to culture plates to stop trypsin. Cells are resuspended and 500 μl solution are transferred onto gel coated culture plates containing 7 ml of fresh M10Gi (for splitting of 1:8), (1:5 splitting: add 4 ml to trypsinized cells and transfer 1 ml).

3.7.6 Freezing of SNL76/7

When SNL76/7 are confluent they can be frozen. Therefore culture medium is discarded and cells are washed with 1x PBS. After removal of PBS 0.7 ml trypsin is added to a 10 cm culture dish and placed into incubator (37°C ; 5%) for 10 minutes. Trypsinized cells are resuspended in 2 ml of M10Gi and transferred to a 15 ml falcon tube. 2.7 ml of freezing medium (with Gi Serum) are added drop by drop to the

suspension. This should be done very slowly. The content is divided into two cryotubes and placed in a Styrofoam box which is then sealed with tape and placed into a -80°C freezer. After two days cryotubes can be moved to a nitrogen tank (-196°C).

Freezing medium:

60% DMEM

20% Gibco Serum

20% DMSO

3.7.7 Preparing feeder cells

All culture dishes must be gelatin-coated before usage. Confluent SNL76/7 plates can be used to make feeder cells. For this purpose, all medium is discarded except 4 ml and 80 μl mitomycin C is added to the culture dishes. After a maximum of 4 hours at 37°C and 5% CO_2 , the supernatant is removed and plates are washed twice with 1xPBS. 1 ml of trypsin is added to each plate and placed at 37° and 5% CO_2 for 5 minutes or until cells are floating around. Cells are resuspended in 5 ml of M10Gi (if more than one plate was trypsinized cells can be pooled with a maximum of three plates) and solution is transferred into a 45 ml falcon. In order to transfer a maximum of cells, plates are washed with additional 10 ml M10Gi. The falcon containing 15 ml cell suspension is centrifuged at 1000 rpm for 7 minutes (Heraeus) and supernatant is discarded. Pellet is resuspended in 1 ml of M10Gi and another 9 ml of M10Gi is added. 50 μl of suspension is added to 10 ml of CASY tone solution and measured with a CASY cell counter (10 μm -30 μm). Cells are diluted till viable cell number reaches 3.5×10^6 . For 24-well plates pipette 0.5 ml/well, for 6-well plates 2 ml/well, and for 6 cm plates use 4ml of suspension. 24-well plates should not be moved too much after pipetting and are directly placed into incubator whilst all other plates should be moved up and down for an equal distribution of feeder cells in the well. Feeder cells can be kept in an incubator (37°C ; 5% CO_2) for up to two weeks and have to be fed at least once a week with M10Gi. Do not use feeder cells that are older than two weeks.

3.7.8 Maintenance of mouse embryonic stem cells (mESCs)

To keep mouse embryonic stem cells undifferentiated they are co-cultured with feeder cells which express leukemia inhibitory factor (LIF).

3.7.9 Thawing of mESCs

Defrosting of ESCs is quite similar to defrosting SNL76/7. Feeder cells must be fed with M10Hi at least 2 hours prior to thawing of mESCs. Cryotubes are removed from liquid nitrogen tank and thawed in a 37°C water bath until only a small icicle is left and are then disinfected with 70% EtOH. The content is transferred to 15 ml falcon tubes and 12 ml of M15Hy are added drop by drop. This should be done slowly and tubes should

be pivoted after every drop. After the first 5 ml are successfully added, pipetting speed can be enhanced to two drops at a time. Tubes are centrifuged at 1000 rpm for 6 minutes and supernatant is sucked off. Cell pellets are resuspended in 1 ml of pre-fed feeder cell supernatant and then transferred back onto feeder cells. To ensure transfer of as many cells as possible wash tubes with another 500 µl of fresh M15Hy. Cell density might be low at first since some cells die during this procedure.

3.7.10 Cultivation of mESCs

ESCs are cultivated at 37°C and 5% CO₂. Medium must be replaced by fresh M15Hy if color switches to orange or yellow. Cells must be split when plate is confluent.

3.7.11 Splitting of ESCs

Feeder cells and ESCs must be fed with fresh M15Hy two hours prior to splitting (feeders 2 ml, ESC 0.5 ml). Medium of the ESCs is sucked off and cells are washed with 1xPBS once. After removal of PBS trypsin is applied to plates (200 µl/24-well; 500 µl/6-well; 1 ml/10 cm) and plates are placed at 37°C and 5% CO₂ for 15 to 20 minutes or till cell adhesion is lost. In case of 24-wells 1 ml of M15Hy medium (for all other wells at least thrice the amount of trypsin used) from previously fed feeder cells is transferred onto ESCs and resuspended well. For splitting 1:2 600 µl of suspension are transferred back onto feeder cells (1:3 400 µl). Plates are cultured at 37°C and 5% CO₂.

3.7.12 Freezing of mESCs

Cells are fed 2 hours prior to freezing. Cells are washed with PBS and trypsinized (200 µl/24-well) and after 15 minutes at 37°C and 5% CO₂ 800 µl fresh M15Hy are added to 24-wells. Cells are resuspended and transferred into 15 ml falcons. 1.8 ml freezing medium is slowly added drop by drop to falcons, after every drop Falcons must be shaken slightly to assure homogenous distribution. After addition suspension is divided into two cryotubes, these are labeled and placed into a Styrofoam box, which is then sealed and put at -80°C for at least two days before transfer of cryotubes into liquid nitrogen tank.

Freezing medium:

60% DMEM

20% HyClone Serum

20% DMSO

3.8 Maintenance of CVPCs

CVPCs are maintained undifferentiated like mESCs on a feeder cell layer of LIF expressing SNL 76/7.

3.8.1 Thawing of CVPCs

CVPCs are thawed exactly as mESCs.

3.8.2 Cultivation of CVPCs

ESCs are cultivated exactly as mESCs.

3.8.3 Splitting of CVPCs

Feeder cells and CVPCs must be fed with fresh M15Hy two hours prior to splitting (feeders 2 ml, ESC 0.5 ml). Medium of the ESCs is sucked off and cells are washed with 1xPBS once. After removal of PBS trypsin is applied to plates (200 µl/24-well; 500 µl/6-well; 1 ml/10 cm) and plates are placed at 37°C and 5% CO₂ for 10 to 15 minutes or till cell adhesion is lost. In case of 24-wells 1 ml of M15Hy medium (for all other wells at least thrice the amount of trypsin used) from previously fed feeder cells is transferred onto ESCs and resuspended well. For splitting 1:2 600 µl of suspension are transferred back onto feeder cells (1:3 400 µl). Plates are cultured at 37°C and 5% CO₂.

3.8.4 Freezing of mESCs

Cells are fed 2 hours prior to freezing. Cells are washed with PBS and trypsinized (200 µl/24-well) and after 10 to 15 minutes at 37°C and 5% CO₂ 800 µl fresh M15Hy are added to 24-wells. Cells are resuspended and transferred into 15 ml Falcons. 1.8 ml freezing medium is slowly added drop by drop to falcons, after every drop Falcons must be shaken slightly to assure homogenous distribution. After addition suspension is divided into two cryotubes, these are labeled and placed into a Styrofoam box, which is then sealed and placed at -80°C for at least two days before transfer of cryotubes into liquid nitrogen tank.

3.9 Embryoid bodies

3.9.1 Making embryoid bodies

24 hours before making EBs mESCs cultured on feeder cells in 24-wells must be split at a ratio of 1:2 because mESCs should be in the log phase of proliferation. Cells are fed 2 hours prior with M15Si and 6-well plates should be coated with gelatine. After 2 hours the medium is discarded and the cells are washed with 1xPBS and 200 µl of trypsin is added to the cells. Plates are incubated at 37°C and 5%CO₂ until cells dissociate. 800 µl of M15Si are added to stop the reaction and suspension is transferred onto gelatin coated 6-well plates containing 2 ml of M15Si in order to separate stem cells from

feeder cells. Feeder cells adhere faster to the gelatine surface of the 6-well plates whereas embryonic stem cells remain nonattached for a longer time period. After 50 minutes of incubation at 37°C and 5% CO₂, supernatant is transferred into a 15 ml falcon. In order to make sure most embryonic stem cells are transferred, 1 ml of M15Si is trickled onto 6-well plates and then transferred to the falcon. Suspension is filled up to 10 ml total volume and 20 µl are transferred into a CASY tube containing 10 ml of CASY ton. The cell number is determined using CASY counter. Suspension is diluted with M15Si to a cell concentration of 4×10^4 cells/ml. 2 ml of this dilution is sufficient for one 10 cm dish of hanging drops.

Sterile culture plates are filled with autoclaved Milli-Q water (a 4mm high water level is enough). 4 ml of cell suspension is transferred into a 6 cm plate. A multi-pipette is set so that 20 µl (equals 800 cells per drop) are dispensed each time. The pipette is loaded and held at a 90° angle to the lid of the culture plate. Drops are placed as close as possible without mixing. Flip lid over and place on top of water filled bottom half. Place carefully at 37°C and 5%CO₂. This time point is referred to as day 0. At day 4.7 the hanging drop cultures have to be transferred onto gelatine coated 10 cm tissue culture plates to allow attachment of the EBs to the culture dishes. 10 cm petri dishes are coated with gelatine 2hours prior to use. Hanging drop cultures are taken out of incubator and 8 ml of M15Si are used to rinse the embryoid bodies from the lid of the culture dish onto the bottom of the petri dish plate. Place dishes at 37°C and 5% CO₂ and do not move for 24hours.

3.9.2 Culture of embryoid bodies

Embryoid bodies must be fed every 3 days with M15Si, medium is only partially replaced (day 7 and 10: 3ml of old medium+5 ml fresh; day 13 to 24: 4 ml old+10 ml fresh, day 25 and thereafter: 5 ml old+12 ml fresh). Partial medium replacement ensures that secreted factors remain in medium at low concentration.

3.9.3 SF9 cell culture

The SF9 cell line was derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*. The SF9 cell line is highly susceptible to infection with *Autographa California* nuclear polyhedrosis virus (AcNPV baculovirus), and can be used with all baculovirus expression vectors. SF9 cells are commonly used to isolate and propagate recombinant baculoviral stocks and to produce recombinant proteins.

3.9.4 Thawing and adaptation of SF9 cells

SF9 cells are kept in liquid nitrogen for storage and stock purposes.

The cells are quickly thawed and resuspended in SF9 II medium supplemented with 20% serum (Gibco), centrifugated at 1000 rpm for 5 minutes. The resulting pellet is resuspended in 5 ml of 20% serum containing medium again, and the cells are transferred to a 50 ml culture flask. The cells are kept in sealed flasks at 28°C.

Once the culture has reached confluence, cells are gently scraped off the surface, collected and centrifugated at 1000 rpm for 5 minutes. The pellet is then resuspended in medium containing 15% serum, and the cells are seeded on a 50 ml culture flask. This procedure is repeated with serum content decreasing by 5% every time the cells are split. When the serum content is below 5%, usually 2% serum is used for maintenance of the cells.

3.9.5 Storage of SF9 cells

For storage, SF9 cells are collected and resuspended in medium containing 20% serum, equal amounts of freezing medium (containing 20% serum and 20% DMSO) are added drop by drop, and the cells are stored in cryo tubes at -80°C for 24 hours. After that they can be transferred to liquid nitrogen.

3.9.6 Infection of SF9 cells

For expression of the desired isoforms of LIF, the cells are seeded at a correct cell number required for infection (4×10^7 per flask in 20 ml medium) and the virus is added according to the desired MOI. Infection is achieved after 96 hours, upon which the cells are visibly bloated and cell lysis sets in.

3.9.7 Protein purification from SF9 cells

After infection, the cells are collected by centrifugation for 5 minutes at 1000 rpm. The supernatant is removed, 5 ml of 1xPBS are added, and the cells are collected by centrifugation for 3 minutes at 1000 rpm. The 1xPBS is discarded and the cells are frozen at -80°C until further use.

The cell pellet is thawed on ice and resuspended in freshly prepared lysis buffer. This is followed by incubation on ice for 15 minutes. The cell lysate is then mechanically disrupted on ice in a douncer, after which it is incubated on ice for 10 minutes. The lysate is then cleared by centrifugation at 14000 rpm and 4°C for 20 minutes. Thereafter, 10 µl of the supernatant are taken for analysis and the rest is distributed equally amongst four 2 ml Eppendorf tubes containing 200 µl of Ni-NTA (nitrilotriacetic) agarose beads (Quiagen) each, which have been equilibrated in lysis buffer. The beads are then rotated at 4°C for 2.5 hours.

After loading, the beads are pelleted at 4°C, 14 000 rpm for 5 minutes. The beads are washed three times with 500 µl of freshly prepared wash buffer by inverting the tube several times and subsequent pelleting of the beads at 4°C, 14 000 rpm for 5 minutes.

Elution fractions E1 and E2 are obtained by adding 200 µl of freshly prepared elution buffer I, followed by shaking at 800 rpm for 10 minutes and subsequent pelleting of the beads at 4°C, 14 000 rpm for 5 minutes, after which the supernatant is collected. Elution fractions E3 and E4 are obtained by adding 200 µl of freshly prepared elution buffer II, followed by shaking at 800 rpm for 10 minutes and subsequent pelleting of the beads at 4°C, 14 000 rpm for 5 minutes, after which the supernatant is collected. The purified protein is stored at 4°C and analyzed on an SDS polyacrylamide gel.

Lysis buffer

50 mM NaH₂PO₄

300 mM NaCl

10 mM Imidazole

adjust pH to 8 using 1 M NaOH

add NP-40 at a dilution of 1:100 directly before use

Wash buffer

50 mM NaH₂PO₄

300 mM NaCl

10 mM Imidazole

adjust pH to 8 using 1 M NaOH

Elution buffer I

50 mM NaH₂PO₄

300 mM NaCl

50 mM Imidazole

Adjust pH to 8 using 1M NaOH

Elution buffer II

50 mM NaH₂PO₄

300 mM NaCl

250 mM Imidazole

adjust pH to 8 using 1M NaOH

All reagents used in protein purification contain protease inhibitors Aprotinin, Leupeptin, and Pepstatin A at a dilution of 1:1000.

If the protein is to be used for activity assays in cell culture, the sample must be freed from salts and imidazole prior to use. This is achieved by dialysing against a semi-permeable membrane as follows:

Preparation of dialysis membrane:

1. 15 cm of a regenerated cellulose tubular membrane (Roth) are rinsed with distilled water.

2. The membrane is placed into a 50 ml Falcon tube containing 50 ml of Solution 1 and heated in a waterbath at 60°C for 3 hours (this removes any traces of heavy metal ions).
3. The membrane is rinsed again with distilled water.

Preparation of Eppendorf tubes:

4. Holes are burnt into the lids of Eppendorf tubes with a heated Pasteur pipette.
5. 400 µl of sample are added per tube.

Dialysis:

6. Pieces of the membrane (~2 cm) are cut off and clamped underneath the lids of the prepared Eppendorf tubes, thereby covering the holes. The rest of the membrane is stored in 40% Ethanol.
7. The tubes are centrifuged upside down at 1000 rpm for 1 minute to test for leakage.
8. If no leakage is visible, the tubes are placed upside down into a beaker containing 1xPBS at a concentration of 1:1000 in relation to the sample amount. Otherwise, repeat step 7.
9. The beaker is left over night at 4°C, stirring.

Solution 1:

1 mM EDTA

2% Sodiumhydrogencarbonate

After dialysis, concentration and purity of the protein are measured with the Nanodrop device.

3.10 SDS Polyacrylamide gel

Isolated proteins are treated with 3x Sample buffer and separated on a denaturing SDS-polyacrylamide gel.

3x Sample buffer:

3 ml Glycerin

0.9 g SDS

3.75 ml of Solution 7

1.75 ml ddH₂O

6 mg Bromphenolblue

- aliquot and store at -20°C
- add 150 µl β-Mercaptoethanol for 850 µl sample buffer aliquot before use

SDS polyacrylamide gel

Solution	15%	12%	9%	6%	Stack
9	5 ml	4 ml	3 ml	2 ml	250 µl
7	2.5 ml	2.5 ml	2.5 ml	2.5 ml	-
8	-	-	-	-	600 µl
H ₂ O	2.45 ml	3.45 ml	4.45 ml	5.45 ml	1.59 ml
TEMED	5 µl	5 µl	5 µl	5 µl	5 µl
10% APS	50 µl	50 µl	50 µl	50 µl	25 µl

Table 1: Reagents for one mini-protean gel (Biorad).

Solution 7:

1.5 M Tris/Cl pH 8.8 (18.17 g Tris)

0.4% SDS (4 ml 10% SDS)

H₂O to 100 ml

Solution 8:

0.5 M Tris/Cl pH 6.8 (6.06 g Tris)

0.4% SDS (4 ml 10% SDS)

H₂O to 100 ml

Adjust pH to 6.8

Adjust pH to 8.8

Solution 9:

30% Acrylamide (30g Acrylamide)

0.8% Bis N,N'-Methylenbisacrylamide (0.8 g Bis N,N'-Methylenbisacrylamide)

H₂O to 100 ml

10x Running buffer:

30g Tris

144g Glycine

10g SDS

H₂O to 1l

Running conditions:

After polymerization of the gel, proteins and a size marker (BioRad low range or high range ladder) are loaded and run in a BioRad Mini Protean Gel chamber, in 1x running buffer, with 25 mA for the first 15 minutes and then 50 mA for 1-2 hours, depending on the percentage of the gel.

3.11 Coomassie staining

SDS polyacrylamide can be analyzed by staining with Coomassie brilliant blue for 30 minutes, followed by incubation with destaining solution over night.

Preparative gels for Mass Spectrometry analysis must be stained with fresh Coomassie staining solution in a glass container. Gel pieces are excised using sterile scalpels (Swann Morton) in a laminar flow hood.

Coomassie brilliant blue

45% (v/v) Methanol

10% (v/v) Acetic acid

0.4% (w/v) Coomassie Brilliant Blue

Destain

4.5% (v/v) Methanol

10% (v/v) Acetic acid

3.12 Western blot analysis

Proteins separated by a denaturing SDS-polyacrylamide gel are transferred to a nitrocellulose membrane (Schleicher and Schuell) using a Biorad semi-dry transfer cell. The blot consists from the bottom upwards of: 6 layers of whatman paper the same size as the gel, one nitrocellulose membrane the same size as the gel, the gel and 6 layers of whatman-paper the same size as the gel. All components are soaked with blotting buffer.

Air bubbles are removed by applying pressure with a Falcon tube. Blotting is carried out at 16 V for 1 hour in a Biorad semi-dry transfer cell.

10x Blotting buffer:

0.48 M Tris

0.4 M Glycine

ddH₂O

Adjust pH to 9.1

After blotting the protein is stained with Ponceau-S for 5 minutes and rinsed with water to assess transfer and to visualize the protein ladder, which can then be marked with a pen.

Ponceau-S

100 mg Ponceau-S

100 ml 1% glacial acetic acid

Antibody incubation and development of the blot:

After blotting, the membrane is blocked with blocking solution for 1 hour. This is followed by three wash steps with 1xTBS/0.1% Tween for 5 minutes each and incubation over night with primary antibody solution. On the following day, the membrane is washed three times with 1xTBS/0.1% Tween for 5 minutes each and incubated with the secondary antibody solution for 1 hour. The membrane is then washed three times with 1xTBS/0.1% Tween for 5 minutes each.

- for an AP conjugated antibody the blot is incubated with AP buffer for 5 minutes

- the blot is incubated with 10 ml of AP buffer with 33 μ l of NBT (added first) and 66 μ l of BCIP (add second)
- the blot is incubated in the dark until bands become visible (5-60 minutes), the reaction is stopped by removing AP buffer and washing with water

Blocking solution:

5% dry milk powder (low fat)

dissolve in 1xTBS/0.1% Tween

Primary antibody solution

5% BSA

dissolve in 1xTBS/0.1% Tween

desired amount of primary antibody

Secondary antibody solution

5% dry milk powder (low fat)

dissolve in 1xTBS/0.1% Tween

desired amount of secondary antibody

NBT

75 mg NBT

700 μ l Dimethylformamid

300 μ l H₂O

AP Buffer:

100 mM Tris/Cl pH 9.5

100 mM NaCl

50 mM MgCl₂

BCIP

50 mg BCIP

1 ml Dimethylformamid

ECL stock solution:

Dilute 20 ml 1MTris in 200 ml H₂O

Add 0.5 ml p-Coumaric acid

Add 1 ml Luminol

Primary antibody	Secondary antibody
α LIF 1:500	anti goat AP 1:10 000

Table 2: Antibodies used for Western blotting.

3.13 Immunofluorescence

Let cells or CBs grow on gelatin coated coverslips in cell culture plates until a proper cell density is reached.

Fixation of cells on coverslips

- Wash cells twice with PBS
- Slowly add EtOH (-20°C) drop wise
- Store cover slips 20' at -20°C in EtOH
- Add 800 μ l dH₂O (4°C) until an EtOH concentration of 70% is reached.
- Store 10' at -20°C
- Cells can now be used for immunostaining or can be stored at 4°C. Cover plates with parafilm.

3.14 Immunostaining:

- Suck off EtOH solution and wash 3x 5' with PBS.
- Dilute primary antibody in PBS during washing steps.
- Drop 100 μ l primary antibody solution on the lid of a 6 well culture plate.
- Take the coverslip out of the washing solution with a forceps and place it upside down on the antibody drop.
- Give wet tissues into the bottom wells of the 6 well plate and put it over the lid.
- Incubate the 6-well plate for 1,5 hours in a wet chamber.
- Remove the coverslips from the lids and wash it 3x 5' with 2 ml PBS in 6-well-plate wells.
- Dilute secondary antibody during washing in PBS.
- Drop 100 μ l of the secondary antibody dilution on the lid of a 6-well plate and place the coverslips upside down onto the drops.
- Incubate for 45' in a wet chamber.

- Wash 1x 10' with 2 ml PBS and a according to instruction manual concentration of DAPI (4',6-diamidino-2-phenylindole).
- Wash 2x 10 min with 2 ml PBS.
- Preheat mowiol to 50°C during washing.
- Cut of a yellow 200 µl Gilson pipette tip and pipette 40 µl mowiol onto a glass slide.
- Put the cover slips upside down onto the mowiol drops.
- Let the preparation dry over night.
- Seal the edges of the coverslips with non-fluorescing nail polish.

Primary antibody	Secondary antibody
α cTnT 1:100	α goat FITC Ab 1:200
α SPARC 1:50	α rabbit TR 1:200

Table 4: Antibodies used for Immunostaining.

3.15 Confocal laser scanning microscope (CLSM)

In contrast to conventional wide field fluorescence microscopes confocal laser scanning microscopes produce images which are focused on an optical plane with selectable depth.

Zeiss LSM 510 META confocal microscope:

After switching on the UV lamp, the microscope, the lasers and the computer the Zeiss LSM 510 META software has to be started. First the correct excitation light wave length and the light emission filters like FITC/TRITC have to be chosen. Switch on UV light and focus on DAPI stained nuclei to find the correct optical plane. Switch from visual mode to computer mode.

Step by step manual:

- Start software LSM 510 META in expert mode.
- Go to "Aquire/Lasers" and turn on required Lasers.
- Put a drop of oil on the cover slip.
- Go to "Aquire/VIS" to activate visual mode.
- Switch "FL on/off" button on and turn on UV-light. Focus on the correct plane by focusing on e.g. DAPI stained nuclei.

- Go to “Aquire/LSM/Scan” for scanning an image.
- In the scan control window choose “Fast XY” to adjust the laser intensity while the image is updated every few seconds.
- After adjustment of the laser intensity choose “Stop/Single” for scanning of a high resolution image.
- Go to “File/Save” as to save the image.
- The format of the pictures is “.lsm” and they can only be opened with the LSM 510META program. For conversion to a different file format choose “Export/tif”.

3.16 Reverse transcriptase polymerase chain reaction

3.16.1 Isolation of mRNA with RNAeasy Mini KIT (Qiagen):

At least 1 confluent plate of a 24-well plate of CVPCs or mESCs is necessary for RNA isolation.

- Suck off medium and wash cells 2x with PBS.
- Add 1ml PBS and scrape off cells.
- Centrifuge 5' at 13.000 rpm with Eppendorf 5415C.
- Discard supernatant and resuspend pellet in 600 µl RLT buffer containing 6 µl β-mercaptoethanol.
- Load lysate onto Qia-shredder column and homogenize at 13.000 rpm.
- Mix lysate with 600 µl 70% RNase free EtOH.
- Load 600 µl of lysate onto RNeasy mini columns, put columns into a 2 ml tube and spin at 13.000 rpm for 15”.
- Discard flow-through and load remaining 600 µl of lysate.
- Wash columns with 350 µl RW1, 15”, 13.000 rpm.
- Wash columns 2x with 500 µl RPE
- Dry spin columns in empty 2 ml tubes.
- Put columns into 1,5 ml eppendorf tubes.
- Elute RNA with 40 µl RNase free water.
- Spin 1', 13.000 rpm.
- Mix 30 µl RNA solution 3.75 µl DNase Buffer and 3.75 µl DNase I. Digest DNA for 30' at 37°C.
- Stop digestion by adding 3.75 µl stop buffer (EDTA), 10' 65°C. Take 1 µl for agarose gel. Store RNA at -80°C.

3.16.2 Reverse transcription:

- Prepare RT-Mix: 60 µl 5x buffer, 30 µl 0,1MDTT, 9 µl RNAsin (RNase out, or Ribolock), 12 µl 10 mM dNTPs.
- Add 1 µl oligo dT.
- Incubate 10', 70°C.
- Leave 3' on ice.
- Spin 15'', 13.000 rpm.
- Add 18,5 µl RT-Mix
- Incubate 2' at 42°C
- Add 1 µl reverse transcriptase and incubate 50' at 42°C.
- Stop reaction with 15' at 70°C.
- Put 5' on ice.
- Centrifuge 2' at 13.000 rpm.
- Store cDNA at -80°C.

3.16.3 Polymerase chain reaction:

- PCR standard reaction mix :
 - 1 µl cDNA
 - 5 µl 10x PCR buffer
 - 1.5 µl 50 mM MgCl₂
 - 1 µl 10 mM dNTPs
 - 0.5 µl forward primer (100pmol/µl)
 - 0.5 µl reverse primer (100pmol/µl)
 - 0.25 µl taq polymerase (1.25 U)
 - 40.25 µl ddH₂O

Standard PCR-program:

Step	Temperature	Time
1	94°C	1 min
2	94°C	45 sec
3	Annealing temperature	45 sec
4	72°C	1 min
5	72°C	5 min
6	4°C	infinite

29 to 35 cycles between step 2 and step 4 were used depending on PCR efficiency.

Used Primer and annealing temperature:

Gene		Primer sequence	Annealing temp.	Cycles	PCR product	
BMP2	Fwd	5' – GTTTGTGTTTGGCTTGACGC – 3'	57.5°C	34	cDNA	719bp
	Rev	5' – AGACGTCTCAGCGAATTTG – 3'				
Desmin	Fwd	5' – TGACAACCTGATAGACGA – 3'	50°C	37	cDNA	390bp
	Rev	5' – TTCTTATTGGCTGCCTGA – 3'			Genomic	1972bp
Gata4	Fwd	5' – GCCTGTATGTAATGCCTGCG – 3'	53°C	31	cDNA	500bp
	Rev	5' – CCGAGCAGGAATTTGAAGAGG – 3'			Genomic	3364bp
GAPDH	Fwd	5' – CGTCTTCACCACCATGGAGA – 3'	55°C	29	cDNA	300bp
	Rev	5' – CGGCCATCACGCCACAGTTT – 3'			Genomic	300bp
Mef2C	Fwd	5' – GGCCATGGTACACCGAGTACAACGAGC – 3'	66°C	34	cDNA	395bp
	Rev	5' – GGGGATCCCTGTGTTACCTGCACTTGG – 3'				
Mesp1	Fwd	5' – AGAAACAGCATCCCAGGAAA – 3'	52°C	32	cDNA	346bp
	Rev	5' – GTGCCTGCTTCATCTTTA – 3'			Genomic	619bp
Nanog	Fwd	5' – AGGGTCTGCTACTGAGATGCTCTG – 3'	60°C	25	cDNA	364bp
	Rev	5' – CAACCACTGGTTTTCTGCCACCG – 3'			Genomic	4793bp
Oct 3/4	Fwd	5' – CCACCTTCCCCTGGCTGGACA – 3'	50°C	37	cDNA	1082bp
	Rev	5' – AGGGCTGGTGCCTCAGTTTG – 3'			Genomic	4475bp
Tropo-myosin α	Fwd	5' – CAAGCGGAGGCTGATAAGAAGG – 3'	55°C	30	cDNA	310bp
	Rev	5' – TGCCTCTCTCACTCTCATCTGC – 3'				
Nkx2.5	Fwd	5' – CACCCACGCCTTTCTCAGTC – 3'	57°C	40	cDNA	513bp
	Rev	5' – TGGACGTGAGCTTGAGCA – 3'				
Sox2	Fwd	5' – AACCCCAAGATGCACAAGTC – 3'	55°C	34	cDNA	202bp
	Rev	5' – CTCCGGAAGCGTGACTTA – 3'				
SPARC	Fwd	5' – GTCCCACTGAGCTGGC – 3'	55°C		cDNA	590bp
	Rev	5' – AAGCACAGAGTCTGGGTGAGTT – 3'				

Table 5: Primer and annealing temperature:

Fwd: Forward Primer

Rev: Reverse Primer

cDNA: Sequence of mRNA PCR product

Genomic: Sequence of genomic DNA PCR product

3.16.4 Agarose gel

1% Agarose gels were used for analysis of PCR products. Agarose gels were stained with ethidiumbromide for 20 min to visualize DNA fragments.

100 ml 1% agarose gel: 1g agarose, 100 ml dH₂O

4 Results:

4.1 CVPCs autonomously differentiate to cardio-vascular cells in hanging drop culture

Analogous to ESCs CVPCs were maintained undifferentiated by cultivation on LIF expressing SNL76/7 feeder cells. 3 CVPC clonal cell lines (B5, G3 and H3) cultivated on feeder cells were trypsinized and transferred to gelatin coated 6 cm cell culture plates for pre-adsorption of the co-cultivated fibroblast feeder cells. Fibroblasts can thereby be removed because they adhere faster than CVPCs to the gelatin coated matrix of cell culture plates. After 50 minutes the supernatant containing solely CVPCs was taken off. 50 20 μ l drops containing 800 CVPCs each were placed onto the lid of a 10 cm culture plate. 3 lids with 50 hanging drops each were established per clone. Cardiac bodies were observed daily by phase contrast microscopy (Figure 1).

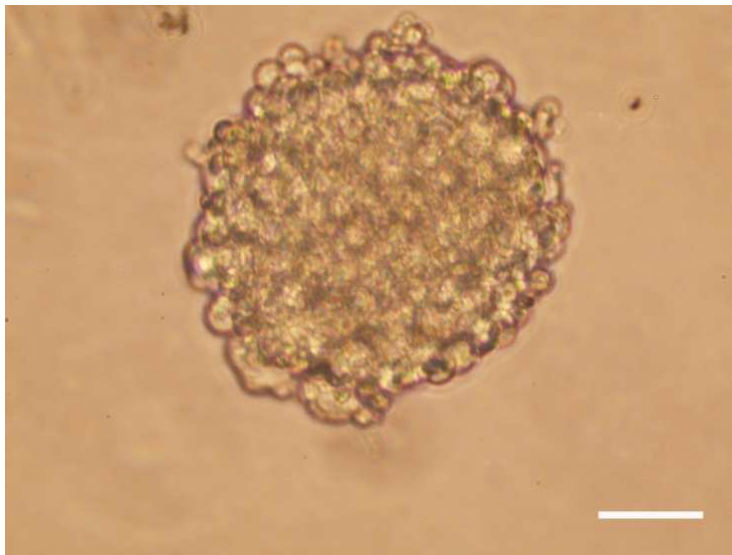


Figure 1: Cardiac body consisting of 800 CVPCs clone H3 24 hours after preparation inside a hanging droplet: The CVPCs assembled at the lowest point of the drop and started to aggregate. Bar=100 μ m.

The lids with the hanging droplets were carefully put onto sterile plates filled with autoclaved double-distilled water and incubated at 37°C for 4 days. The cells in the drops formed a compact sphere which was named 'cardiac body' (CB) because of the morphological and methodical similarity to embryoid bodies (Figure 2). At day 4 the cardiac bodies were rinsed off into a gelatin coated 10 cm cell culture plate.

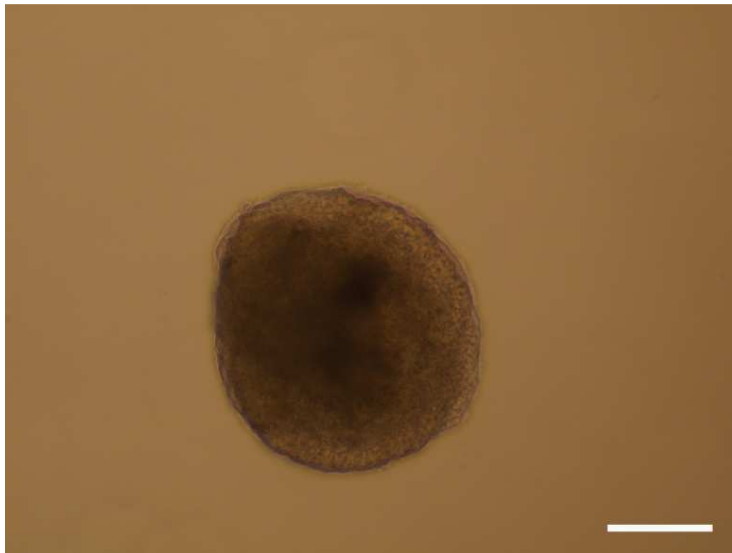


Figure 2: Cardiac body of CVPC clone H3 at day 5 after transfer to a cell culture plate: A compact sphere has formed which will adhere to the gelatin coated plate surface. Bar=100 μm .

Cardiac Bodies adhered to the gelatin coated surface of the cell culture plates within 24 hours. The CBs started to spread and cell divisions and growth of the CBs was observed. Cysts developed after day 6 which were apparent in sporadic non-adhered swimming CBs as bubbles. Monolayers of endodermal cells were observed in the periphery of the CBs. At day 12 beating cardiomyocytes were observed in the center of the CBs. Smooth muscle cell contractions were visible at day 15 in B5 and G3 CBs. Cardiomyocytes and smooth muscle cells developed into fibers (Figure 3). Beating cardiomyocytes and smooth muscle cells were counted daily until day 26. Medium was exchanged every third day.

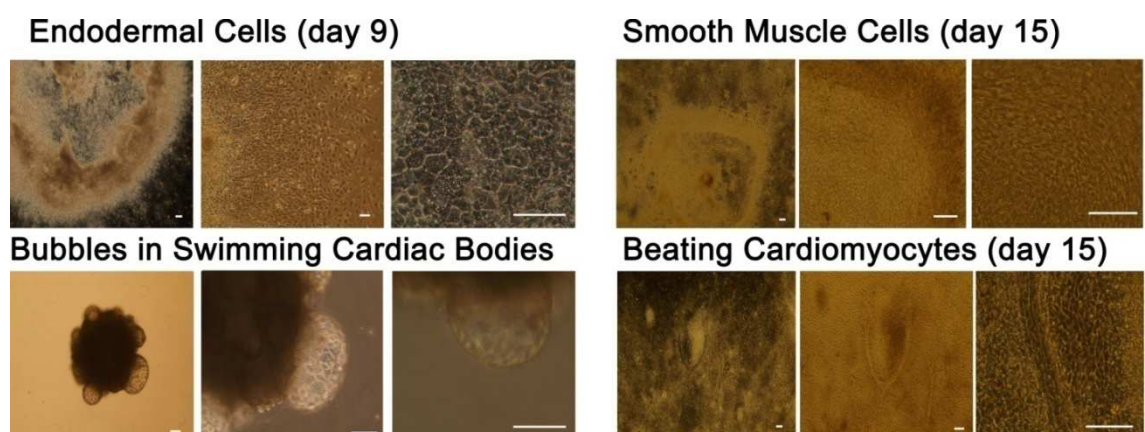


Figure 3: Terminal differentiation of Cardiac Bodies: Three different cardiovascular cell types were observed in cardiac bodies attached to the plate, contracting smooth muscle cells, contracting cardiomyocytes and endodermal cells. Furthermore bubbles of putative vascular endothelial cells were present in all cardiac bodies. Bars=100 μm .

All three clones B5, G3 and H3 differentiated into both beating cardiomyocytes (CMCs) and contracting smooth muscle cells (SMCs) at a different rate. H3 preferentially differentiated into beating cardiomyocytes with 90-100% of observed cardiac bodies containing CMCs (Figure 4) between day 16 and 20, whereas B5 developed cardiac bodies with 50-100% SMCs (Figure 5) between day 16 and 20.

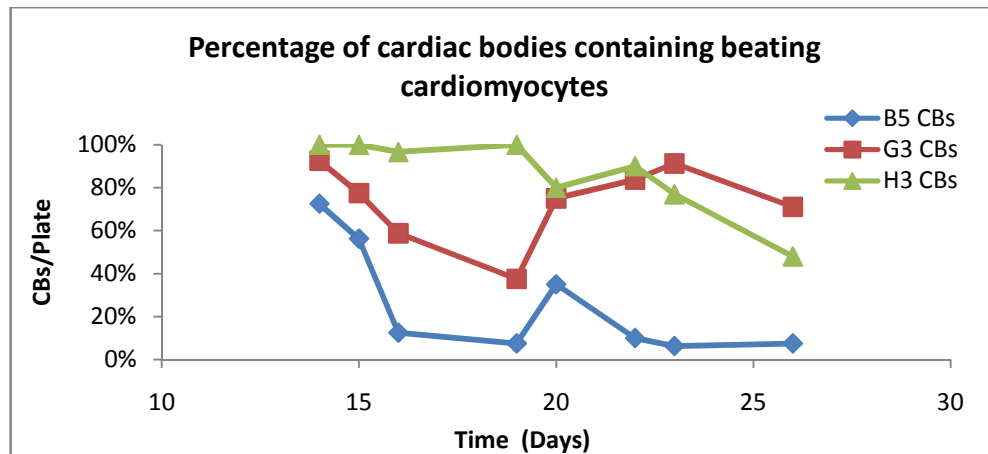


Figure 4: Percentage of cardiac bodies containing beating cardiomyocytes: The percentage of CBs containing beating CMCs was determined for CVPC-clone B5 (B5 CBs), G3 (G3 CBs) and H3 (H3 CBs). H3 showed the highest percentage of CBS with active CMCs, B5 the lowest.

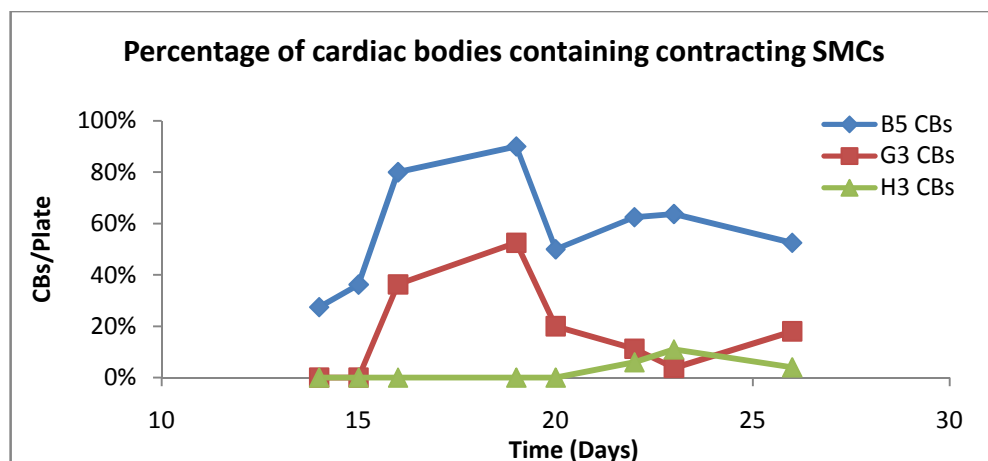


Figure 5: Percentage of cardiac bodies containing contracting smooth muscle cells: The percentage of CBs containing beating CMCs was determined for CVPC-clone B5 (B5 CBs), G3 (G3 CBs) and H3 (H3 CBs). B5 showed the highest percentage of CBs containing active SMCs, H3 the lowest.

Only cells belonging to the three major types of cardiac cells were apparent: cardiomyocytes, vascular endothelial cells and smooth muscle cells. Cells which typically arise in embryoid bodies e.g. skeletal muscle cells or neurons were not observed indicating that CVPCs have a limited differentiation potential.

4.1.1 Activity of CMCs and SMCs in cardiac bodies is antagonistic

When the activity of CMCs and SMCs was compared it became obvious that the activity of SMCs and CMCs is antagonistic (Figure 6A-C). In B5-CBs SMC contractions were dominant, whereas in H3-CBs CMC contractions were dominant. In B5-CBs the high CMCs activity at day 13 was replaced by the later onset of SMCs activity around day 16. In G3-CBs the activity of CMCs was low but increased when SMCs activity decreased after day 20. In H3-CBs CMC activity was predominant and less than 10% of CBs showed contracting SMCs.

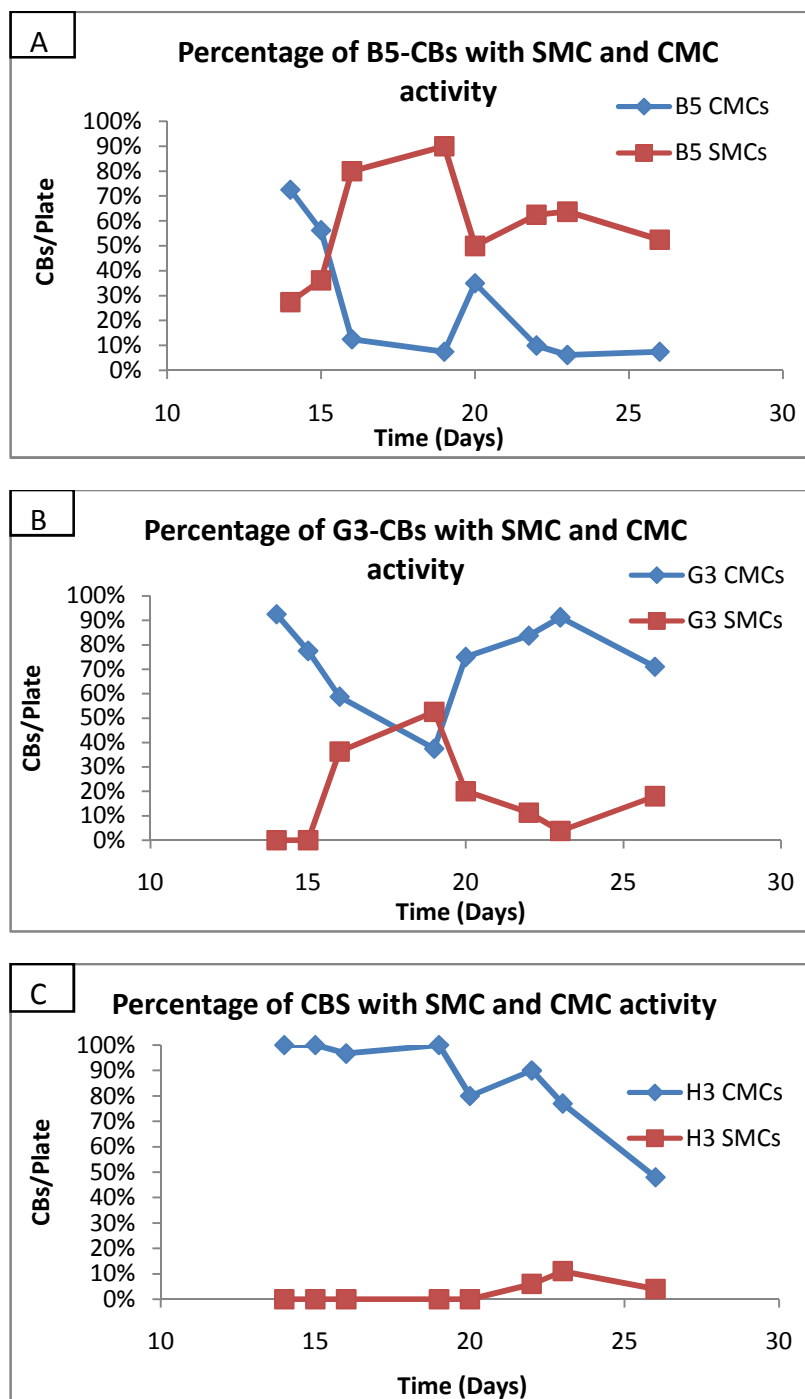


Figure 6: Comparison of active CMCs to SMCs in CBs: A: CBs of clone B5, B: CBs of clone G3, C: CBs of clone H3.

The CBs of all three clones were identical in morphology and CMCs and SMCs were both present. The only difference between the clones was the number of contracting SMCs and CMCs. High activity of both types of muscle cells at the same time was not observed.

4.1.2 Cardiac bodies express cardiac troponin T

To verify that CMCs in CBs express cardiomyocyte-specific proteins CVPC-clone A5 cardiac bodies (CB) from day 21 containing beating CMCs were ethanol fixed and immune-stained for cardiac troponin T. Troponins form complexes consisting of 3 proteins called troponin I, troponin C and troponin T which are part of the myofibrils in cardiomyocytes. Troponin T binds to tropomyosin, troponin C binds calcium ions and cardiac troponin I binds actin. CBs were stained with goat anti-cardiac troponin T antibodies (α cTnT) diluted 1:200. FITC-conjugated anti goat antibodies served as a secondary antibody. cTnT was present in all examined CBs. cTnT showed the typical striated pattern (Figure 7).

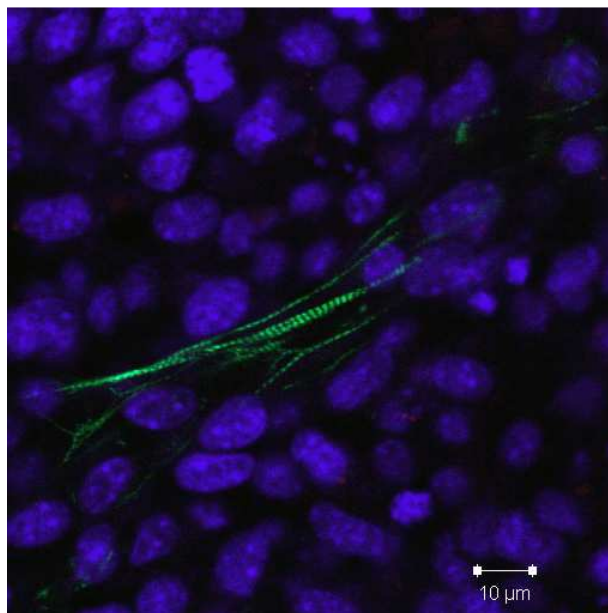


Figure 7: Immunofluorescence of cTnT in A5-CBs: cTnT (green) staining shows the typical striated pattern in myofibrils of cardiomyocytes in A5-CBs at day 21. An oil immersion 63x objective lens was used. Nuclei were stained blue by DAPI.

Corresponding to clusters of beating CMCs observed in CBs, clusters of myofibrils containing striated cTnT fibers were searched for. Immunofluorescence showed expression of cTnT in clusters similar in size to beating CMC clusters observed in CBs (Figure 8).

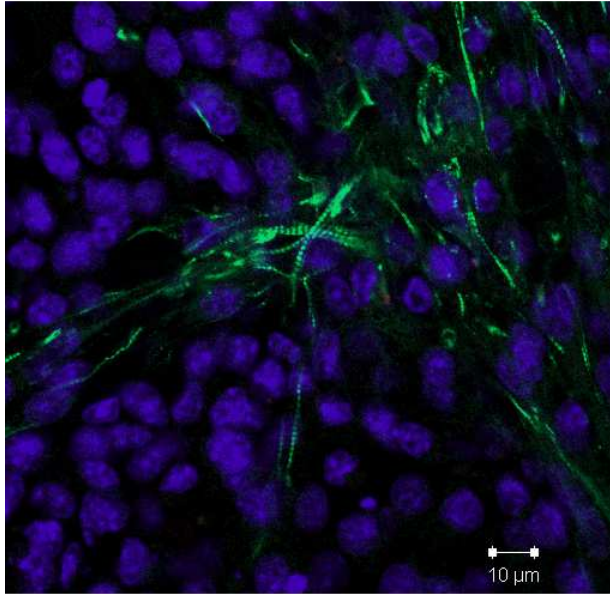


Figure 8: Clusters of myofibrils containing cTnT in A5-CBs at day 21: cTnT (green) is present in myofibril-clusters corresponding in size to areas of beating CMCs which were observed by phase contrast microscopy in CBs. Nuclei were stained blue by DAPI.

4.2 CVPCs autonomously differentiate to cardiomyocytes in vitro

ESCs readily differentiate to CMCs in EBs, but not when seeded on cell culture plates. To examine the differentiation potential of CVPCs in vitro, CVPCs were seeded on gelatin coated culture plates at densities ranging from 100.000 to 300.000 cells per well in 6-well cell culture plates. Cells were incubated at 37°C and 5% CO₂ and the medium was exchanged regularly. Active contracting CMCs were observed in all experiments and were counted daily (Figure 9, Table 1). In contrast to CBs no smooth muscle cells emerged in these experiments.

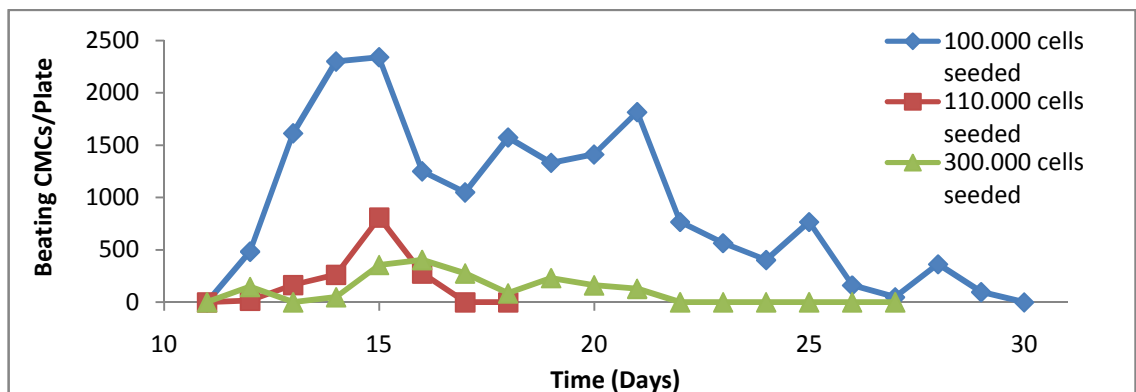


Figure 9: Beating aggregates of CMCs at different seeding densities: CVPCs were seeded onto a gelatin coated 6-well plate at different densities 100.000, 110.000 and 300.000 CVPCs per plate respectively. CVPCs differentiated and beating aggregates of CMCs were observed and counted daily beginning at day 11.

Table 1: Influence of the quantity of seeded cells on the differentiation of CVPCs to CMCs

Experiment	Clone	Seeded cells	Onset of activity	Maximal number of beating CMCs	Time of activity (Days)
A	H3	100.000	day 11	2339	17
B	H3	110.000	day 13	807	4
C	H3	300.000	day 13	403	9

H3: CVPC clone H3; Seeded cells: Seeded CVPCs clone H3 per 6-well culture plate; Maximal number of beating CMCs: Highest number of beating CMC aggregates counted on a single day.

In the experimental setup an optimum with a maximal number of CMCs was reached when 100.000 CVPCs clone H3 were seeded (table 1, experiment A). A higher quantity of active CMCs resulted in longer activity and higher longevity of CMCs. The experiment showed that CVPCs have to be seeded sub-confluent at a certain concentration on cell culture dishes to efficiently differentiate to beating CMCs. The amount of seeded cells influences the differentiation of CVPCs to active CMCs.

4.3 Influence of serum concentration on cardiomyogenesis

To investigate which effect the fetal bovine serum-concentration in the medium has on the differentiation of CVPCs, cells were seeded onto gelatin coated cell culture plates and fed with medium containing 5%, 10% and 15% bovine fetal serum, respectively. In a small scale experiment after 4 days of cultivation of 300.000 cells of CVPC-clone H3 in a 24-well cell culture plate, 8000 cells were seeded into plastic rings containing 300 μ l medium, 3 rings per gelatin coated 6-well cell culture plate. After 4 days the rings were displaced. The beating aggregates of CMCs were counted daily (Figure 10A and B).

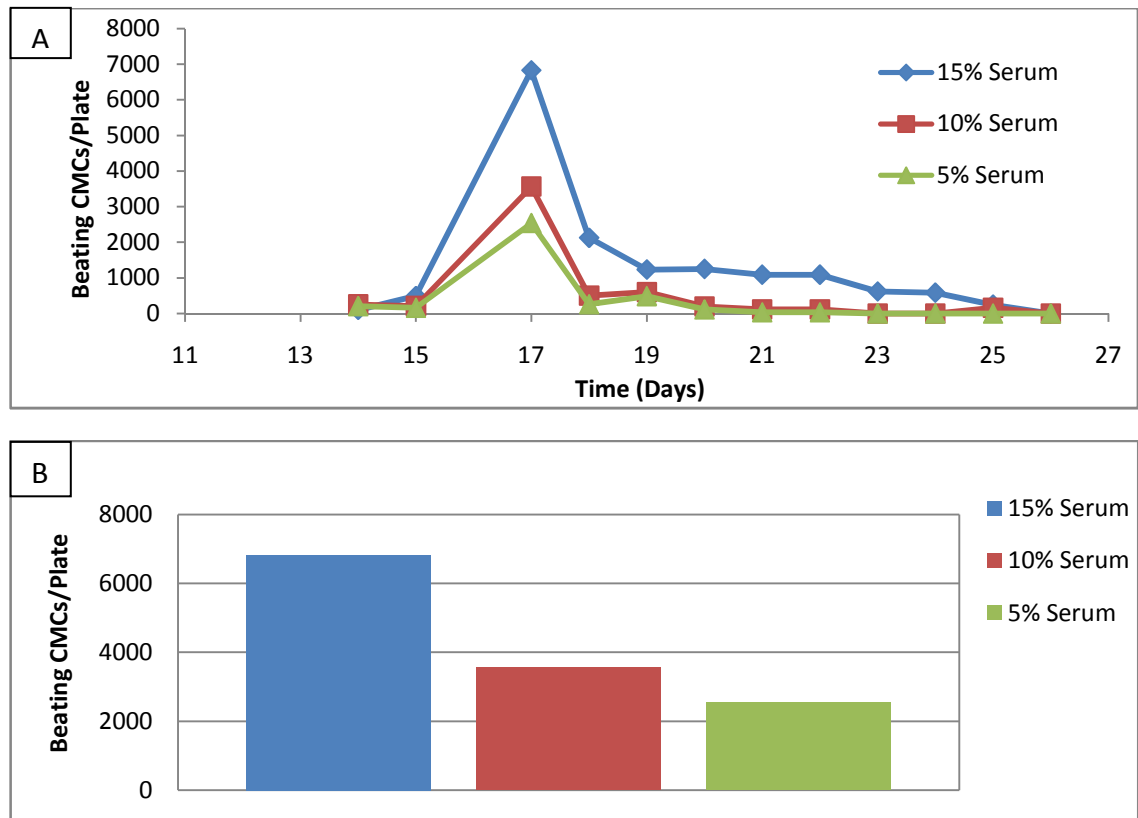


Figure 10: Serum concentration during CVPC in vitro differentiation influences quantity, maximal number and longevity of active CMCs:

A: Quantity of beating areas of CMCs from day 14 to day 26 when fed with medium containing 15%, 10% and 5% FBS.

B: Comparison of the number of beating CMCs in CVPCs at the maximum of activity on day 17 when fed with medium containing 15%, 10% and 5% FBS.

The medium containing the highest concentration of 15% v/v fetal bovine serum was the most favorable for efficient differentiation of CVPCs to CMCs. Furthermore, a higher concentration of FBS caused a higher longevity of beating CMCs. Fetal bovine serum does not have a defined composition, and in this setup it was not determined which factors in the serum are influencing cardiomyogenesis.

4.4 Effects of leukemia inhibitory factor on CVPC differentiation

LIF is usually used to maintain mESCs or CVPCs undifferentiated. However, Bader et al (2001) showed that in EBs, LIF promotes proliferation of cardiomyocytes in a developmental stage-dependent manner (see introduction page 14). Further experiments in the Weitzer laboratory demonstrated a cardiomyogenesis-promoting effect of M-LIF on CVPCs as well (Wiedner 2008). LIF expressed in SF9 insect cells which are ovarian cells of *Spodoptera frugiperda*, showed a higher cardiomyogenesis-promoting impact on mESCs than recombinant LIF produced in bacteria which lacks eukaryotic modification of proteins. Therefore LIF was expressed in SF9 cells and tested on CVPCs.

4.4.1 Expression of M-LIF and D-LIF in SF9 insect cells

The LIF-gene codes for three different isoforms of LIF: Truncated (T-LIF), matrix associated (M-LIF) and diffusible LIF (D-LIF), which are generated by alternative splicing. M and D-LIF have almost the same size and sequence but differ in their N-terminal amino acid-sequence, a putative leader sequence.

D-LIF: MKVLAAGIVPLLLL...

M-LIF: MRCRIVPLLLL...

Although M-LIF and D- LIF are both secreted, they may have different effects as was shown by former studies (Conquet et al. 1992; Pasteiner 2006). Truncated LIF remains inside the cell.

The SF9- Baculovirus system was chosen because in contrast to bacterial expression systems insect cells also generate posttranslational modifications in expressed proteins such as phosphorylations, myristylations, palmitylations, and glycosylations. LIF is known to be heavily glycosylated, thus glycosylation will have an effect on activity and function of LIF. SF9 cells were infected with viruses containing M-LIF and D-LIF expression vectors. SF9 cells were infected with baculoviruses containing M-LIF and D-LIF expression vectors constructed by Waltraud Pasteiner (Pasteiner 2006). A viral plaque assay done by Marc Wiedner showed that both viruses exhibit a titer of 2.2×10^5 plaque forming units (PFU) per ml. A multiplicity of infection (MOI) of 0.01PFU/cell was used for both infections. 4 days after infection cells were harvested and stored at -80°C. Infected cells are bigger in size and appear bloated and granular (Figure 11).

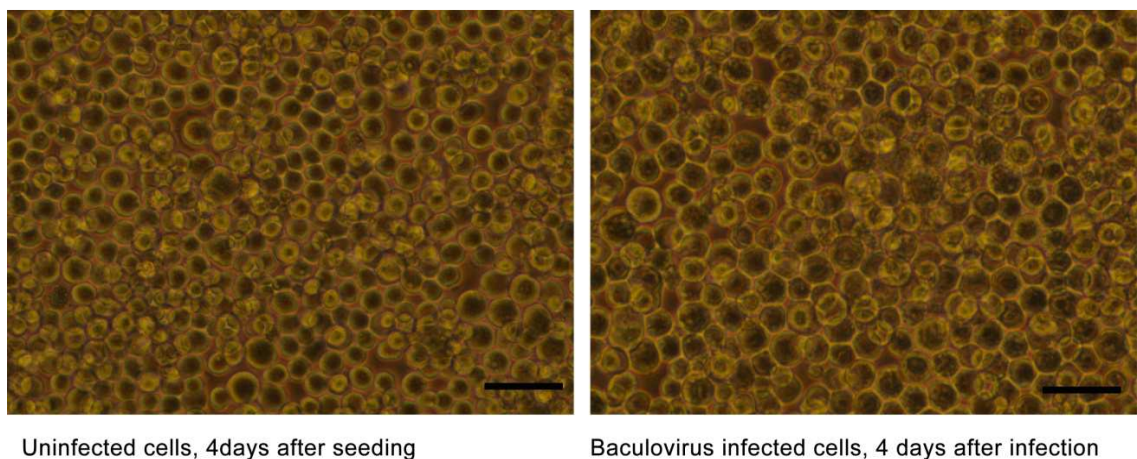


Figure 11: SF9-cells before and after baculovirus infection. Bar=10 μ m

4.4.2 Purification of M-LIF and D-LIF

SF9 cells were lysed and the M-LIF and D-LIF proteins were purified using Ni^{2+} -NTA agarose beads. Expressed M-LIF and D-LIF proteins are histidine-tagged and contain a stretch of 6 histidines at the C-terminus which binds to Ni^{2+} . The LIF proteins were eluted with imidazole and fraction 1-4 were collected; the first two were eluted with 50 mM imidazol and the last two with 250 mM imidazol, respectively. The collected fractions were analysed on a 12% SDS-polyacrylamide gel (Figure 12). M-LIF and D-LIF are 30-40 kDa in size, depending on the degree of glycosylation. Unglycosylated D-LIF and M-LIF have a calculated mass of 22.29 kDa and 22.16 kDa, respectively.

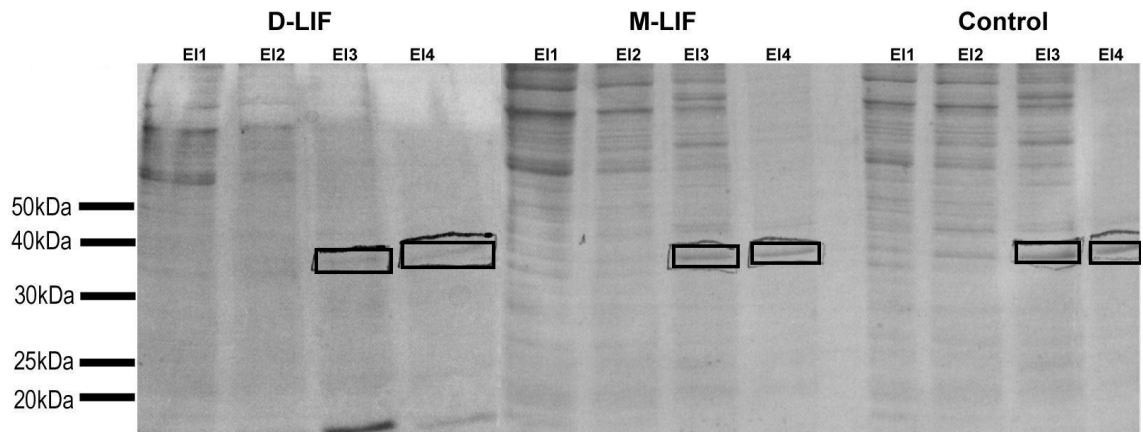


Figure 12: SDS-PAGE of D-LIF, M-LIF and control (uninfected SF9-cells) Ni^{2+} -NTA agarose fractions.

On the coomassie-stained SDS-PAGE no defined band could be assigned to LIF, because the control showed the same pattern as M-LIF and D-LIF. To verify the presence of LIF in the elution a western blot with α LIF-antibody was performed (Figure 13).

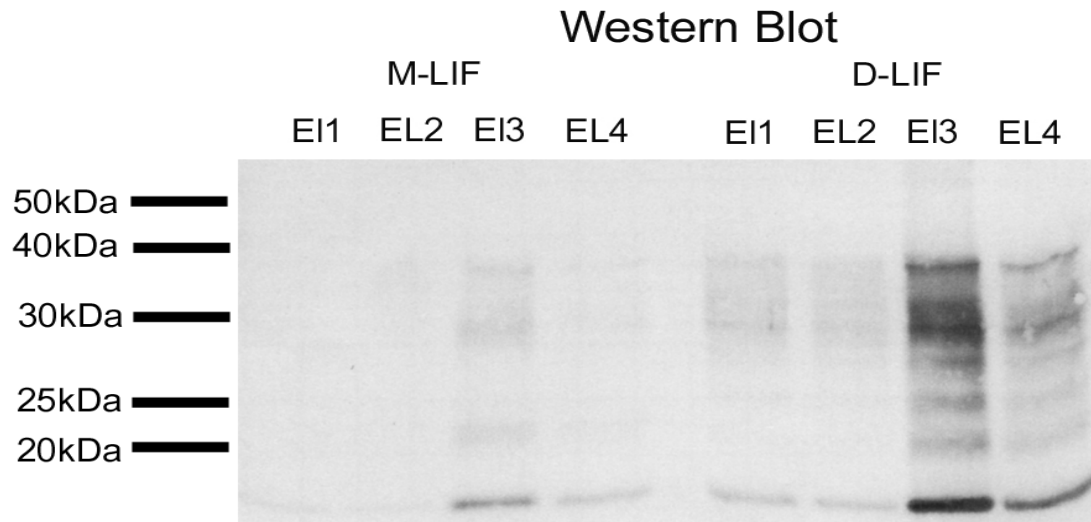


Figure 13: Western blot of M-LIF and D-LIF SDS-PAGE (α -LIF 1:500): The differentially glycosylated forms of LIF range from 40 kDa to 22 kDa a truncated form of LIF appears at 17 kDa.

On the western blot, fraction 3 and 4 where 250 mM imidazole was used for the elution, contained the highest amount of LIFs. The blot showed the presence of M-LIF and D-LIF with differing levels of glycosylation between 22 and 40 kDa in size. D-LIF fractions contained the highest concentration of LIF. Furthermore, a truncated form of LIF was present at 17kDa. Before usage of M-LIF and D-LIF for further experiments in cell culture the LIF containing fractions were dialysed in order to get rid of heavy metal ions and imidazol.

4.4.3 LIF promotes cardiomyogenesis in CVPCs

To investigate the effect of LIF isoforms on cardiomyogenesis during CVPCs differentiation CVPCs were treated with 100 ng/ml M-LIF, D-LIF and recombinant LIF (R-LIF) and as a control with the extraction of uninfected SF9 cells (SF9), respectively and compared to untreated CVPCs (CT). Clones B5 (Figure 14A and B) and H3 (Figure 15A and B) were seeded onto gelatin coated cell culture dishes and treated with LIFs or controls for 4 days. Afterwards, the medium was exchanged and differentiating CVPCs were observed by phase contrast microscopy. Beating areas of CMCs were counted daily.

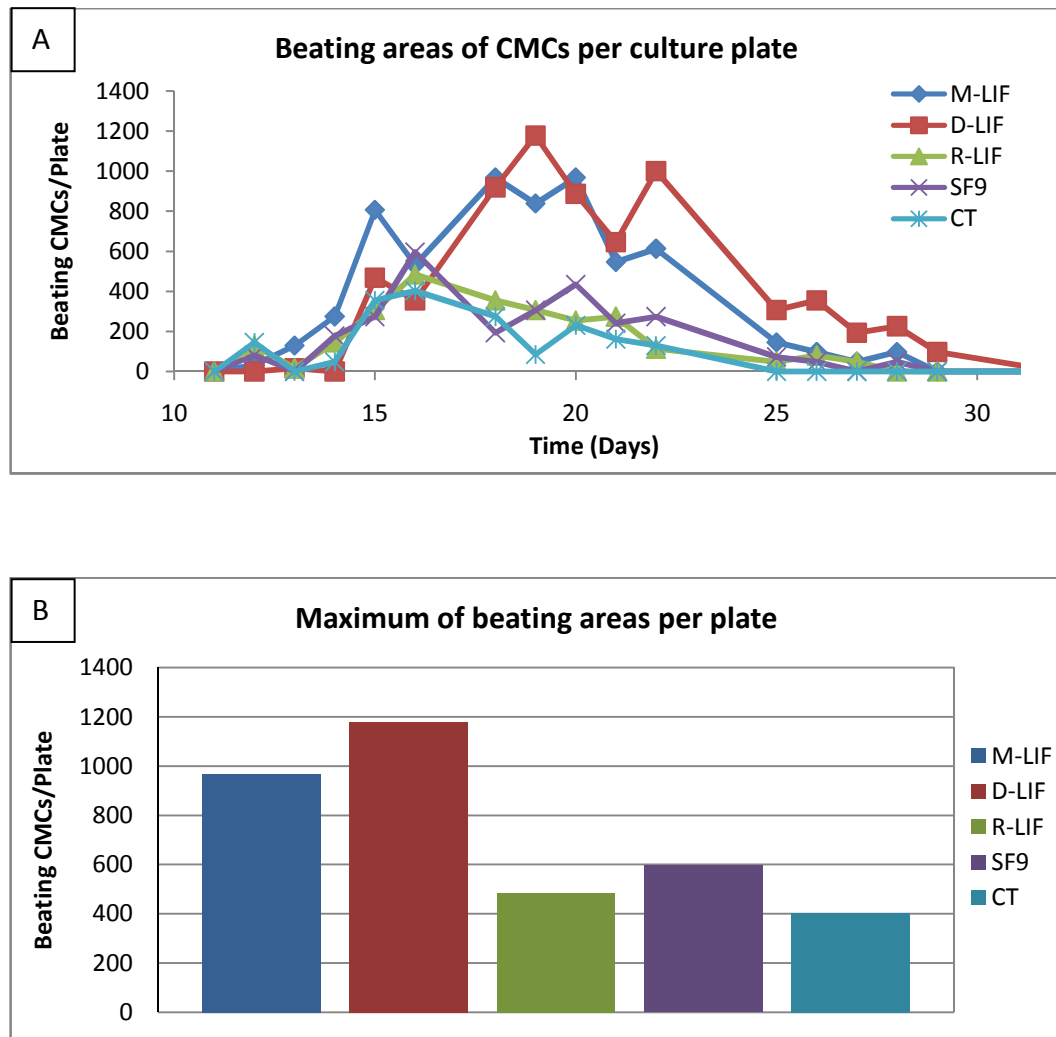


Figure 14: Beating areas of cardiomyocytes after LIF-promoted differentiation of CVPC clone H3. 1×10^5 CVPCs clone H3 were seeded onto a gelatin coated 6-well plate and treated with 100 ng/ml M-LIF, D-LIF, R-LIF or the control extraction (SF9) or left untreated (CT) for 4 days.

A: Comparison of number of beating CMCs for each day of activity.

B: Comparison of the maximum of beating CMCs which occurred at different days, respectively.

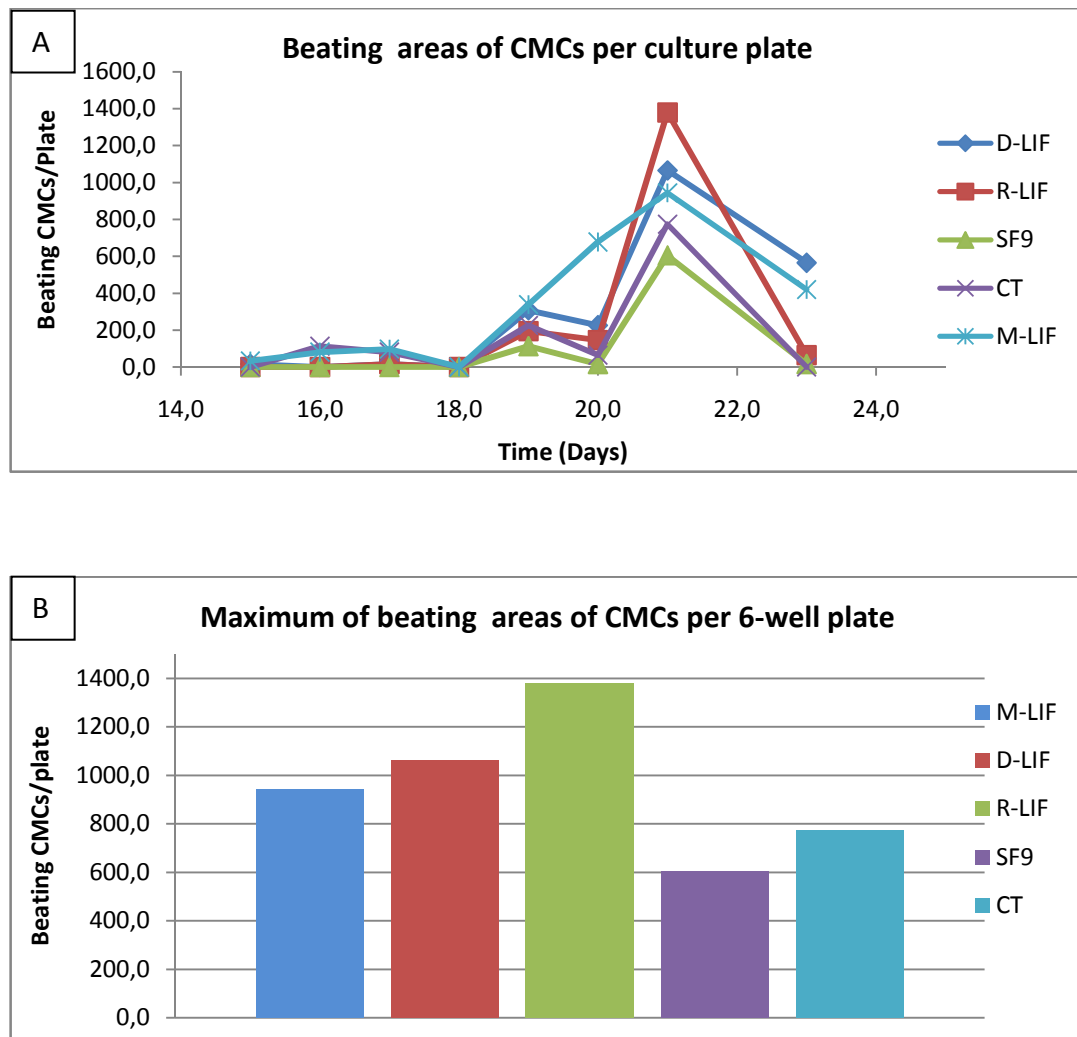


Figure 15: Beating areas of cardiomyocytes after LIF-promoted differentiation of CVPC clone B5. 1×10^5 CVPCs clone B5 were seeded onto a gelatin coated 6-well plate and treated with 100 ng/ml M-LIF, D-LIF, R-LIF or the control extraction (SF9) or left untreated (CT) for 4 days.

A: Comparison of number of beating CMCs for each day of activity.

B: Comparison of the maximum of beating CMCs at day 21.

In H3 M-LIF and D-LIF but not R-LIF showed a cardiomyogenesis promoting effect when compared to SF9 and CT. In B5 all LIFs promoted cardiogenic differentiation.

4.5 Expression of stem cell and cardiomyogenic markers in CVPCs and cardiomyocytes

4.5.1 Stem cell markers Oct3/4, Nanog, Sox2 and mTERT are expressed in CVPCs

2 CVPC clones (G3, H3) were maintained undifferentiated on SNL76/7 feeder cells and the mRNA of clones G3 and H3 was isolated and analyzed by a semi-quantitative RT-PCR (Figure 16A-C). The markers Oct3/4 and Nanog are both transcribed in CVPCs.

Oct3/4 and Nanog are critical stem cell transcription factors which are also expressed in ESC and indicate pluripotency. Telomerase, the enzyme which maintains the telomeres of linear chromosomes in eukaryotes, is only transcribed in stem cells and especially in ESC at high levels. In somatic stem cells mTERT is found at lower levels. In comparison to ESC CVPC clone H3 expresses low levels of mTERT. Sox2 (sex determining region Y)-box 2 which is essential for mESCs self renewal is expressed in CVPC clone H3 as well. In summary the main transcription factors Oct3/4, Sox2 and Nanog which build the transcriptional network of self renewal in mESCs are present in CVPCs, too.

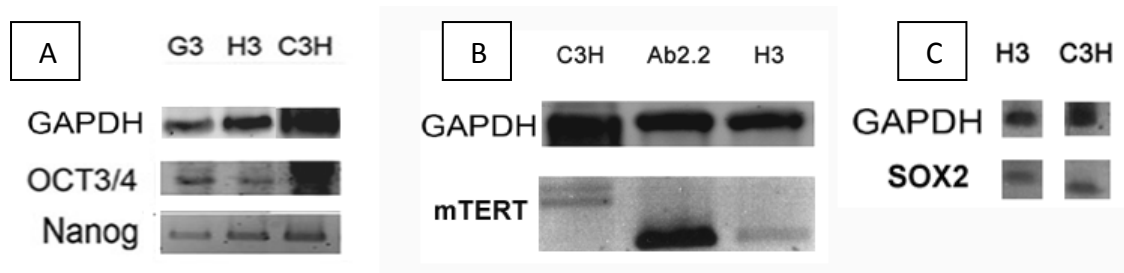


Figure 16: RT-PCR of mRNAs transcribed in CVPCs:

A: Oct3/4 and Nanog mRNAs are transcribed in CVPC clone G3 and H3. As a control PCR genomic mouse DNA (C3H) was used.

B: mTERT (murine telomerase reverse transcriptase) is expressed in clone H3. As a control mESCs mRNA (AB2.2) and genomic DNA (C3H) including introns was used. CVPC clone H3 transcribes mTERT at lower levels than mESCs AB2.2.

C: Sox2 is expressed in clone H3. As a control PCR genomic mouse DNA (C3H) was used.

4.5.2 Transcription of cardiac transcription factors is up-regulated during CVPC

differentiation

During differentiation CVPCs should upregulate cardiogenic transcription factors. CVPC differentiation was induced by cultivation on gelatin-coated culture plates and treatment with 100 ng/ml M-LIF for 4 days. mRNA of beating CMCs was isolated 16 days after M-LIF treatment. Semiquantitative RT-PCR showed that cardiac transcription factors Nkx2.5, GATA4 and muscle cell markers Tropomyosin and MEF2C and Desmin were upregulated in CMCs (Figure 17). Isl1 is a LIM-homeodomain transcription factor which is expressed in cardiac progenitor cells of the second heart field during embryogenesis. Isl1 was found to be expressed in CVPC and at higher levels in CMCs.

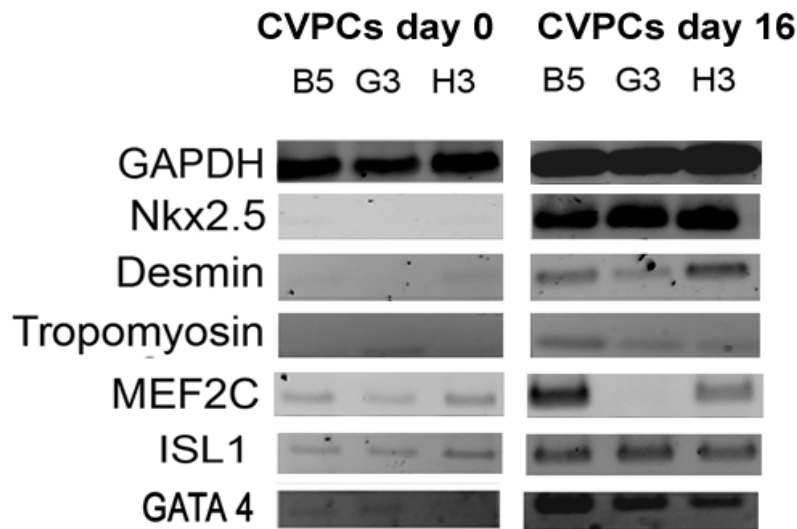


Figure 17: Cardiac transcription factors are up-regulated during CVPC differentiation:
Comparison of mRNA expression in clone B5, G3 and H3 undifferentiated CVPCs and differentiated CVPCs day 16.

4.5.3 Cardiac transcription factor Nkx2.5 expression is up-regulated within 48 hours in differentiating CVPCs

To determine the onset of expression of the cardiac transcription factor Nkx2.5 on translational level, western blots of differentiating CVPCs were done. CVPC clone H3 was seeded on gelatin-coated culture plates at a concentration of 1×10^5 cells per 4 ml in 6-well plates. Differentiating cells were harvested every 24 hours for blotting. Sample protein concentrations were measured with a NanoDrop 1000 UV/VIS fluorospectrometer and diluted to equal concentrations. For western blotting primary α Nkx2.5 antibodies and secondary alkaline phosphatase conjugated antibodies were used (Figure 18). After 48 hours of differentiation Nkx2.5 proteins were detected for the first time. Between day 2 and 6 Nkx2.5 concentration was rising and on day 7 a slight reduction of Nkx2.5 protein was seen. Nkx2.5 mRNA is not present in undifferentiated CVPCs but Nkx2.5 translation is activated within 48 hours of differentiation. The quick up-regulation of the heart specific transcription factor Nkx2.5 demonstrates the immanent cardiomyogenic differentiation potential in CVPCs.

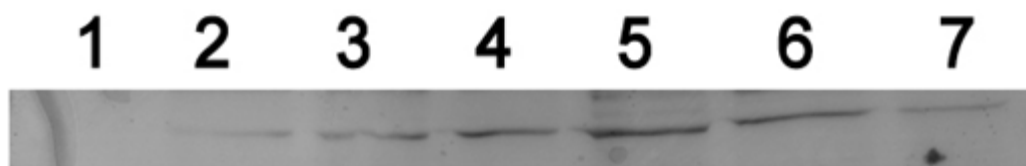


Figure 18: Nkx2. Western blot day 1 to 7 (1-7). Nkx2.5 expression begins at day 2 and is up-regulated during day 2 to 6 and slightly down-regulated at day 7.

4.6 Effects of SPARC and BMP2 on CVPC differentiation

SPARC is an essential protein which promotes cardiac development in embryoid bodies (Stary et al. 2005). Furthermore, Martina Stary showed that in EBs BMP2 promotes cardiogenesis and that SPARC and BMP2 synergistically induce expression of Nkx2.5. Therefore, SPARC and BMP2 may promote cardiogenesis in CVPCs as well.

4.6.1 SPARC is expressed in CVPCs

Again, CVPC differentiation was induced by cultivation on gelatin-coated culture plates and treatment with 100 ng/ml M-LIF for 4 days. The mRNA of differentiated CVPCs containing beating CMCs was isolated 16 days after M-LIF treatment. To determine whether SPARC and BMP2 are transcribed in undifferentiated CVPCs and differentiated CVPCs or not, a semi-quantitative RT-PCR was done. RT-PCR showed that SPARC mRNA is expressed in both, CPVCs day 0 and day 16. BMP2 is slightly upregulated in differentiated CVPCs (Figure 19).

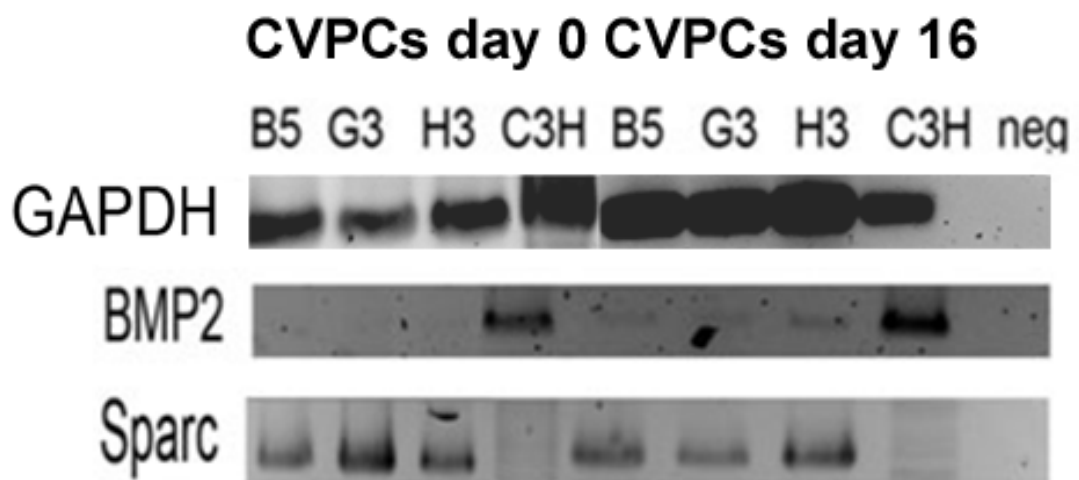


Figure 19: RT-PCR of SPARC and BMP2 in 3 CVPC clones at day 0 and day 16 of differentiation. SPARC is expressed at day 0 and day 16 and BMP2 expression becomes apparent at low levels in differentiated CMCs.

To detect where the SPARC protein is present in CVPCs, differentiating CVPC aggregates with beating CMCs were ethanol fixed at day 16 and immunostained with SPARC antibodies (Figure 20). As secondary antibodies Texas red anti-rabbit were used and nuclei were stained with DAPI. SPARC filled vesicles were found throughout the cytoplasm of CVPCs but not in the nucleus (blue).

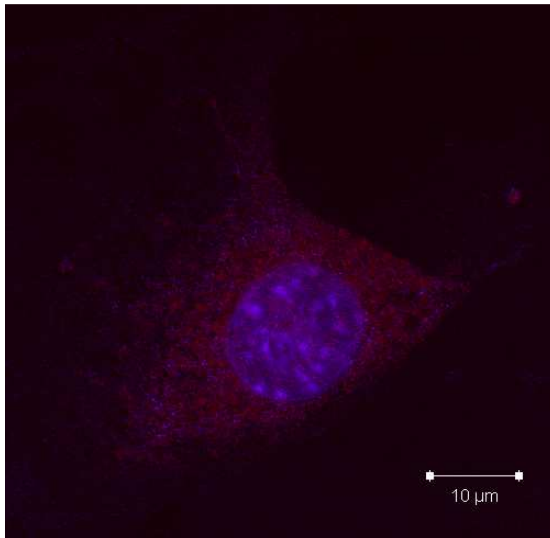


Figure 20: Vesicles-like structures in differentiated CVPCs contain SPARC: SPARC labeled with Texas red secondary antibodies is present in the cytoplasm. Nucleus is stained blue with DAPI.

4.6.2 SPARC promotes cardiomyogenesis in CVPCs in vitro

If SPARC induces cardiomyogenesis in CVPCs like in EBs, treatment with recombinant SPARC should promote cardiomyogenesis in CVPCs. In a newly developed differentiation procedure 6-well cell culture plates were gelatin-coated and 3 plastic rings per well, cut out of Gilson pipette tips, were glued to the plates with sterile Vaseline. The rings were confined water-proof wells with a volume of 500 μ l in which cells can be treated separately from the rest of the well. 8000 undifferentiated CVPCs clone H3 in 300 μ l medium (M15Hy) were seeded into the rings and treated with 2 μ g/ml SPARC for 4 days or left untreated as a control. 8000 CVPCs in a ring equates to seeding 110.000 CVPCs into a 6-well culture plate. After 4 days the rings were displaced to use the whole volume of the well to prevent starvation by lack of medium. Afterwards, the medium was exchanged every second day. Beating CMCs were observed and counted daily. The onset of beating CMCs was at day 16 at the same time in SPARC treated cells and untreated cells, but the quantity of beating CMC was 2-fold higher in SPARC treated cells at day 20 (Figure 21A) and 3-fold higher on average between day 16 and 32 (Figure 21B).

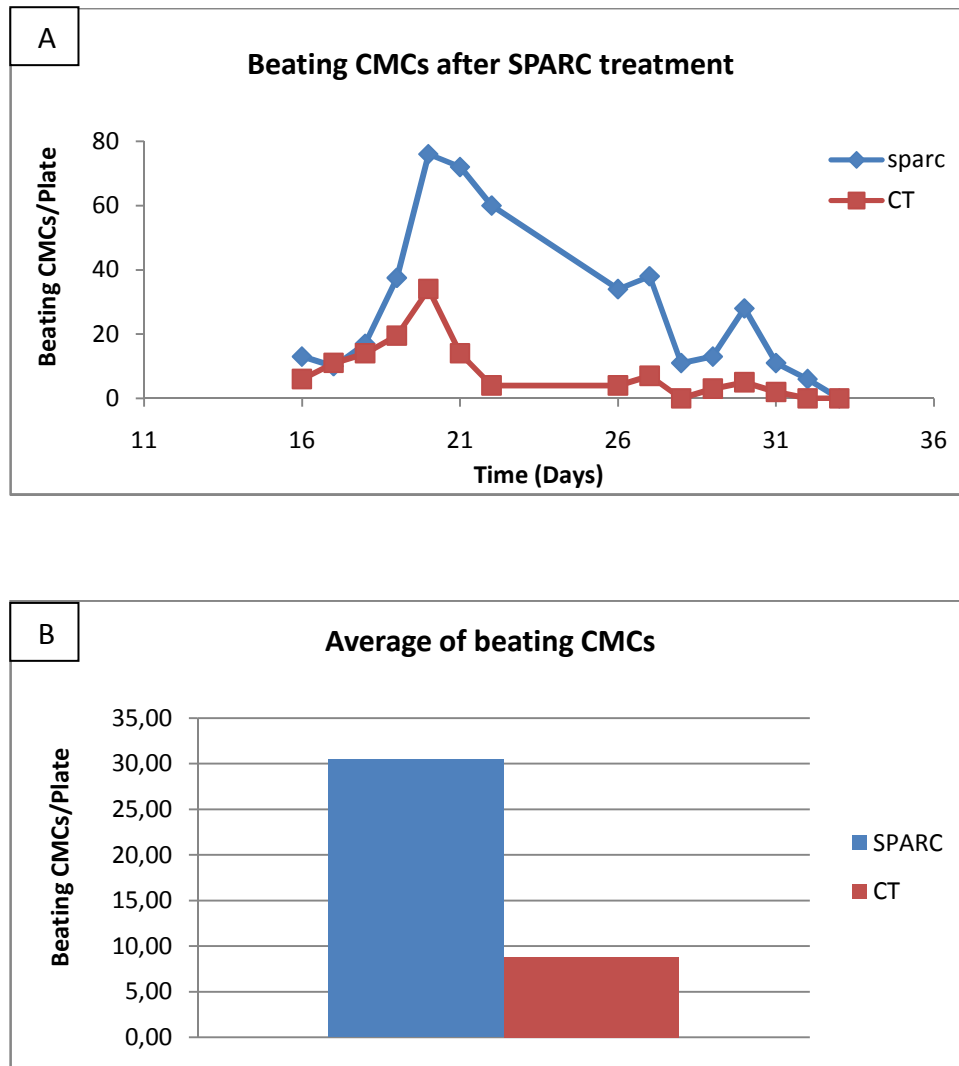


Figure 21: SPARC promotes cardiomyogenesis in CVPCs:

A: Comparison of quantity of beating areas of CMCs in untreated (CT) and SPARC treated CVPCs (SPARC) from day 16 to day 31.

B: Average of beating areas of CMCs in untreated (CT) and SPARC treated cells (SPARC) between day 16 and day 32.

In a second experiment 3×10^5 CVPCs clone B5 and G3 were seeded on gelatin-coated 6-well plates and treated with M-LIF (100 ng/ml) for 4 days. Afterwards 1×10^5 of the cells were treated with BMP2 (100 ng/ml), SPARC (2 μ g/ml) and SPARC plus BMP2 for 11 days (Figure 22). SPARC treated cells beat 2 days prior to cells treated with M-LIF only. BMP2 (bone morphogenetic protein 2) a factor which promotes cardiomyogenesis in embryoid bodies did not induce cardiomyogenesis in CVPCs in monolayers and CVPCs remained morphologically unchanged. BMP2 inhibited SPARC induction of cardiomyogenesis when cells were treated with both SPARC and BMP2.

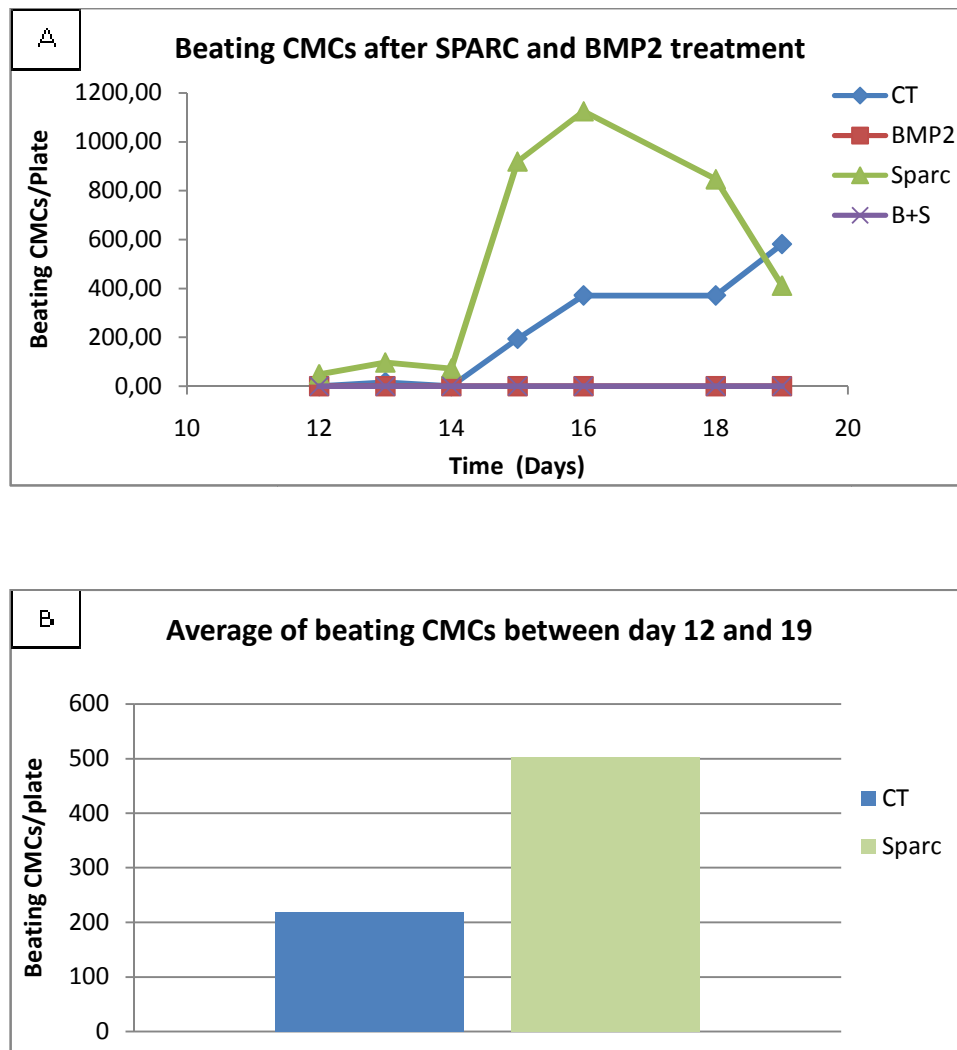


Figure 22: Influence of SPARC and BMP2 on CVPCS differentiation:

A: Comparison of quantity of beating areas of CMCs in untreated (CT), BMP2 treated (BMP2), SPARC treated CVPCs (SPARC) and BMP2 and SPARC treated CVPCs from day 12 to day 19 after seeding per 6-well culture plate.

B: Average of beating areas of CMCs in untreated (CT) and SPARC treated cells (SPARC) between day 12 and day 19 after seeding.

If SPARC promotes cardiomyogenesis, inhibition of SPARC should hamper cardiomyogenesis. Therefore, 1×10^5 CVPCs were seeded in gelatin-coated 6-well plates and treated with SPARC or α SPARC antibodies for 4 days. Beating cells were counted after day 14 and compared to untreated cells (CT). CVPCs treated with α SPARC antibodies showed a delayed onset of activity of beating CMCs when compared to CT and SPARC (Figure 23A). A peak of beating CMCs was reached at day 18 regardless of treatment. SPARC treated wells contained more CMCs (1740) than untreated (1510) and untreated wells more than α SPARC (1387). On average, SPARC treated CVPCs differentiated at a greater extent to beating CMCs than untreated CVPCs and CVPCs treated with α SPARC antibodies had the lowest quantity of beating CMCs (Figure 23B).

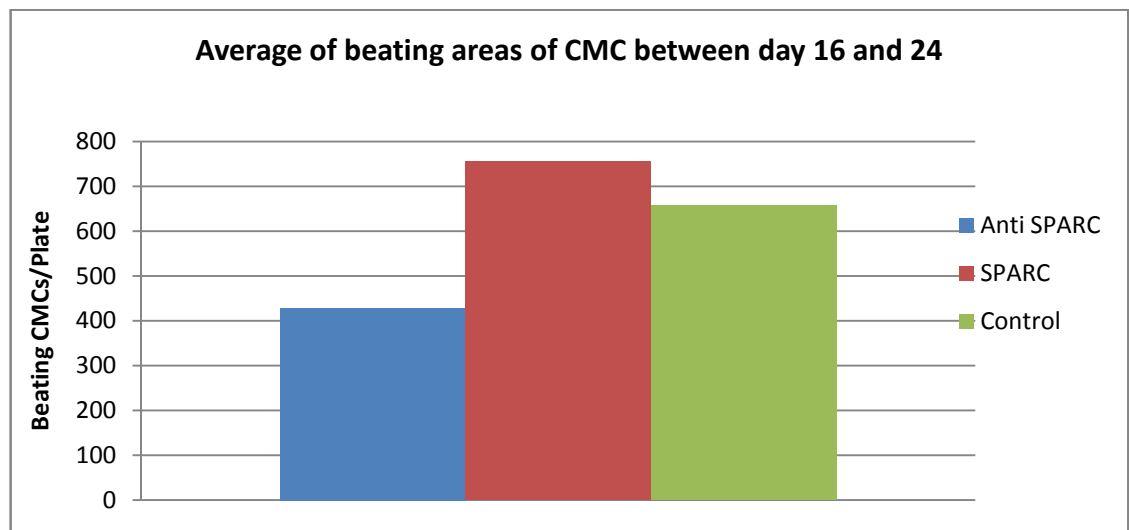
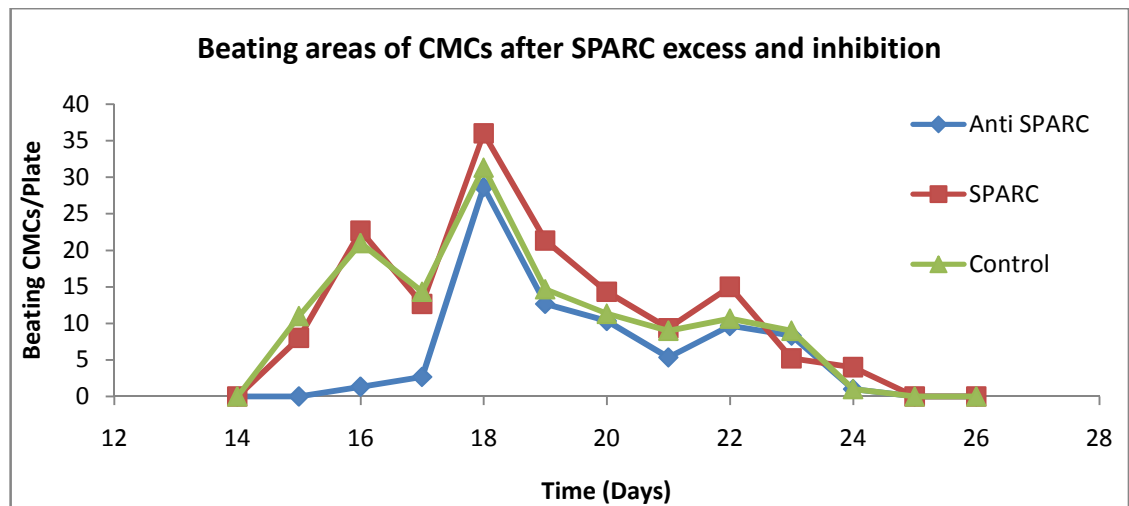


Figure 23: Temporary SPARC inhibition hampers cardiomyogenesis:

A: Comparison of quantity of beating areas of CMCs in untreated (Control), SPARC treated (SPARC) and SPARC antibodies treated CVPCs clone H3 (Anti SPARC) from day 14 to day 26.

B: Average of beating areas of CMCs in untreated (Control), SPARC treated cells (SPARC) and SPARC antibodies treated CVPCs clone H3 (Anti SPARC) between day 16 and day 24.

4.6.3 SPARC induces cardiac transcription factor expression indirectly

During differentiation of CVPCs, cardiac transcription factors have to be expressed for cardiomyogenesis. To see if cardiac marker genes transcription is induced by SPARC, CVPCs were treated with SPARC (2 μ g/ml) and mRNA was isolated 0, 2, 4, 6 and 12 hours after treatment for a semi-quantitative RT-PCR. As a control, CVPCs treated for 12 hours with BMP2 inhibiting cardiomyogenesis was used (Figure 24).

Mesp1 a helix-loop-helix protein which is expressed in all cardiovascular precursor cells, was down-regulated within 2 hours in SPARC and BMP2 treated CVPCs. Desmin a muscle specific type-III-intermediary filament was not upregulated within 12 hours.

SPARC was strongly expressed in all cells regardless of treatment. GATA4 was stably expressed in SPARC treated cells but down-regulated in BMP2 treated cells. The early cardiac marker Nkx2.5 was not expressed in SPARC or BMP2 treated cells within the first 12hours. No short term up-regulation of cardiac marker genes was observed.

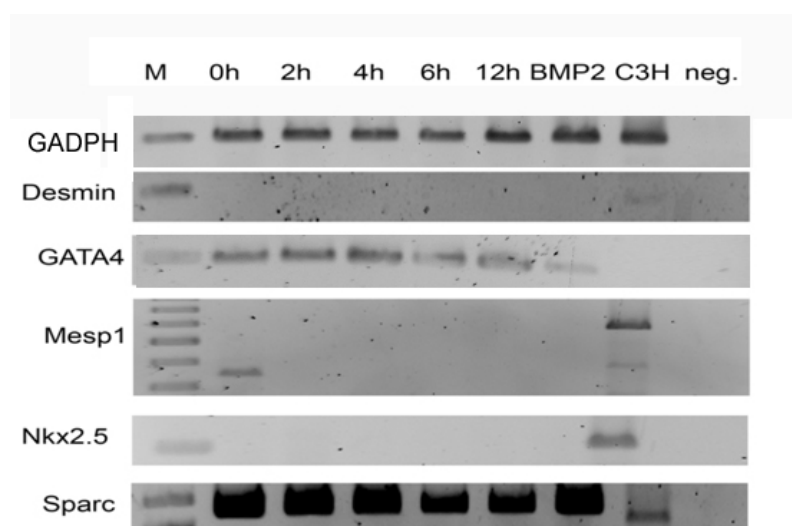


Figure 24: Cardiac marker gene expression in CVPCs during SPARC induced differentiation: mRNA was isolated 0h, 2h, 4h, 6h, and 12h after seeding. BMP2 treated cells were harvested after 12h. Myocardial transcription factor GATA4 was down-regulated in BMP2 treated cells.

Because Nkx2.5 expression is mandatory for cardiomyogenesis and Nkx2.5 is present in CMCs at day 16, but not within the first 12 hours of differentiation, (Figure 25) an experiment with a larger time scale was designed. 3×10^5 CVPCs were seeded and treated with M-LIF or left untreated for 4 days. At day 4, 60.000 cells were re-seeded in gelatin-coated 6-well culture plates and treated with SPARC (0.5 $\mu\text{g/ml}$) and BMP2 (100 ng/ml). mRNA was isolated after 24 hours (day 1), 48 hours (day 2), and 96 hours (day 3) and analyzed by semi quantitative RT-PCR (Figure 13). M-LIF and SPARC induced strong Nkx2.5 expression at day 2. In untreated cells and BMP2 treated strong Nkx2.5 expression was observed 24 hours later at day 3.

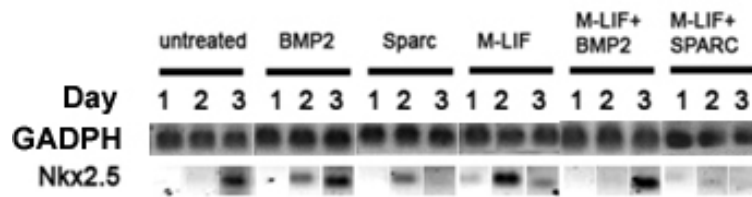


Figure 25: Nkx2.5 expression in differentiating CVPCs: Nkx2.5 is upregulated 24 hours earlier in SPARC or M-LIF treated CVPCs in comparison to BMP2 and untreated CVPCs.

The 2 SPARC differentiation experiments indicate that Nkx2.5 is indirectly activated by SPARC after 24 hours. Adding SPARC in excess accelerates Nkx2.5 transcription and promotes cardiomyogenesis in CVPCs.

4.7 Activating the Wnt pathway by GSK3 inhibition keeps CVPCs undifferentiated

How SPARC promotes cardiomyogenesis is unclear. SPARC physically interacts with ILK1 (Barker et al. 2005). ILK1 is known to inhibit GSK3 (Dedhar 2000), thereby activating the canonical Wnt pathway. Recently, there was evidence that Wnt is an essential regulator of cardiac progenitor cell differentiation (Cohen et al. 2008). Especially the canonical Wnt pathway promotes differentiation of mesodermal progenitors into CMCs in EBs (Kwon et al. 2007). Furthermore, Wnt signaling promotes Nkx2.5 expression and early cardiomyogenesis in mouse embryonic carcinoma stem cells (Liu et al. 2009). All these recent experiments indicate that SPARC may indirectly activate or contribute to activation of Nkx2.5 via ILK/GSK3-linked activation of the Wnt-pathway. To check if the Wnt-pathway induces cardiomyogenesis in CVPCs, the Wnt-pathway was activated by GSK3 inhibition. Therefore, CVPCs were treated with CHIR99021, which is a potent chemical specifically inhibiting GSK3. 3×10^5 CVPCs clone H3 were seeded on 6-well plates and treated with CHIR99021 (1.4 $\mu\text{g/ml}$) for 4 days. A higher quantity of CVPCs was seeded because CVPCs were fed with medium containing 15% horse serum which is of lower quality than bovine serum and the optimal CVPC concentration for differentiation is higher than in FBS. Several differentiation experiments showed that seeding 3×10^5 CVPCs is the best density for autonomous cardiomyogenesis of CVPCs in horse serum (data not shown). Beating CMCs were observed from day 14 until day 26 in untreated but not in CHIR99021-treated CVPCs (Figure 26). CVPCs treated with CHIR99021 remained undifferentiated with a small and round morphology.

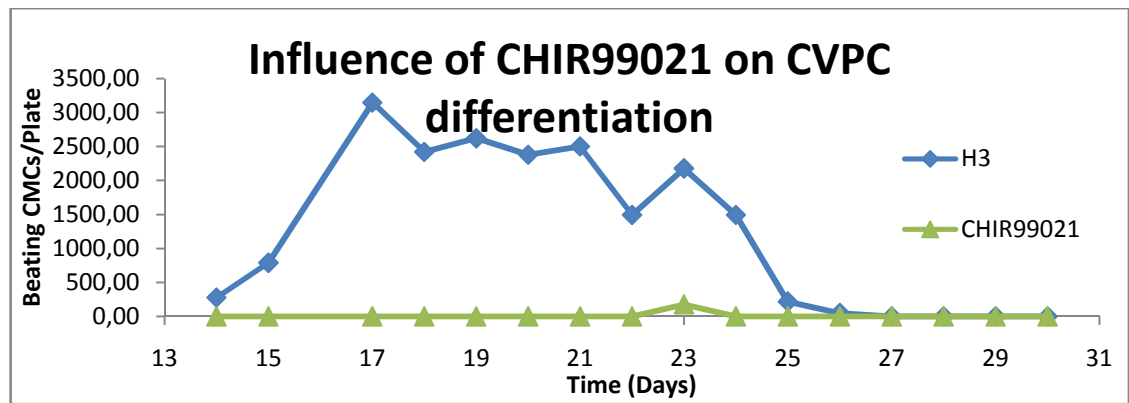


Figure 26: CHIR99021 inhibits differentiation in CVPCs. Untreated CVPCs clone H3 (H3) efficiently differentiated to CMCs whereas CHIR99021 treated CVPCs (CHIR99021) remained undifferentiated.

5 Discussion

5.1 Cultivation of CVPCs

The CVPCs were isolated from the heart tissue of neonatal mice. Neonatal hearts express LIF (Robertson et al. 1993) and LIF together with other factors maintains mESCs undifferentiated. This led to the idea that CVPCs can be cultivated on LIF expressing SNL 76/7 fibroblast feeder cells. It turned out that when cultivated on feeder cells CVPCs remain undifferentiated and proliferate for at least 60 passages without losing their differentiation potential (data not shown). DMEM and fetal bovine serum were sufficient to feed CVPCs and common mammalian cell culture incubators and conditions were used. CVPCs have to be cultivated sub-confluent and splitting is necessary every 3 days. Careful cryo-conservation in liquid nitrogen does not change the characteristics of CVPCs. The morphology of CVPCs differs in size and shape when compared to that of mESCs. CVPCs appear smaller in size and they do not adhere to each other when cultivated on feeder cells.

5.2 Spontaneous in vitro differentiation potential of CVPCs

The first in vitro differentiation experiment with cardiac bodies led to the name CVPCs. In mESCs pluripotency can be demonstrated in vitro with embryoid body experiments where mESCs spontaneously differentiate into every cell type of the early embryo except trophoectoderm. In cardiac bodies however, CVPCs spontaneously differentiated to a restricted number of cell types. Cells of the cardiovascular system, endodermal cells, contracting smooth muscle cells (SMCs), and beating heart cells (CMCs), were observed and endothelial-like cells formed bubbles on the periphery of CBs. No other cell types which develop in embryoid bodies like neuronal cells and skeletal muscle cells were seen. Thus, the cardiac body experiment shows morphologically the limited differentiation potential of CVPCs in vitro. Interestingly, in CBs of different clones CMCs and SMCs developed at different rates. This may be due to differences between the clones or simply to differences in experimental handling. The fact that the more CMCs the less SMCs were active and vice versa, may be a result of changing conditions during the experiment. As CVPCs keep proliferating during differentiation, the cell density is rising, more cytokines and fewer nutrients are present and the number of cells undergoing apoptosis is rising. Furthermore, the composition of different cell types is changing.

The detection of cTnT by immunofluorescence microscopy verified the presence of CMCs in CBs. cTnT was present within myofilaments in the typical striated pattern. Clusters of cTnT also correspond to the beating interwoven networks and clusters of CMCs which were observed by immunofluorescence microscopy in CBs. Smooth

muscle cell actin, a SMC marker, was also detected by immunofluorescence microscopy recently (Hoebaus, 2009). Hence, CVPCs differentiate to CMCs and SMCs which are part of the cardiovascular system. The existence of other cell types in CBs, like the putative endothelial cells, has to be confirmed by further immunofluorescence experiments.

Monolayer differentiation experiments with CVPCs on gelatin-coated culture plates also resulted in efficient differentiation to beating CMCs. In contrast to mESCs, CVPCs differentiated to CMCs when seeded sub-confluent. These experiments showed that CVPCs differentiate autonomously without any treatment to beating CMCs within 12 days. However, no SMCs were observed. Efficient and autonomous differentiation to CMCs was shown for all 11 CVPC clones (Hoebaus, 2009). Depending on the quantity of seeded cells different amounts of beating cardiomyocytes were observed. Furthermore, the more beating CMCs were present the earlier was the onset of CMC activity. Seeding 1×10^5 cells of clone H3 resulted in the highest number of CMCs. Seeding more cells led to less CMC development and seeding fewer cells did not result in any beating CMCs. This indicates that there is an ideal cell density needed for efficient cardiomyogenic differentiation. Reasons for this may be factors like cell-cell interactions and concentration of cytokines or nutrients in the medium. One difference between monolayer experiments and CBs was, when the CVPCs were seeded in monolayers at a low density, in contrast to CBs cell-cell interactions were not existent but formed when the cells proliferated and became confluent. If cells are seeded confluent or nearly confluent no CMCs develop in monolayer experiments. Because in both CBs and monolayer experiments CMCs arise efficiently, it can be concluded that cell-cell interaction has a minor impact on cardiomyogenic differentiation of CVPCs. Factors secreted by differentiating CVPCs or factors present in the medium might be the key regulators of the cardiomyogenic differentiation process.

5.3 Influence of fetal bovine serum on differentiation

Fetal bovine serum is the part of blood plasma which remains after the coagulation and centrifugation of fetal bovine blood. It is a growth supplement used for cell culture and it is produced by many different manufacturers. The main component is the protein bovine serum albumin (BSA) and it contains a lot of other proteins and cytokines like BMPs which influence the differentiation of cells. The composition is not defined and differs between origins and manufacturers. To investigate which impact the concentration of our FBS from the company Hyclone has on the differentiation efficiency of CVPCs, CVPCs were differentiated in media with different FBS concentrations. The experiment showed that higher concentrations of FBS promoted cardiomyogenesis of CVPCs and enhanced the longevity of the resulting CMCs. This effect can be a direct consequence of factors in the FBS inducing cardiomyogenesis or a consequence of FBS-feeding by promoting growth, proliferation and survival of

CVPCs and thereby stimulating cardiomyogenesis indirectly. Because the FBS composition is not defined and changes even from batch to batch of one company, it is difficult to determine which factors in the FBS are responsible for the cardiomyogenesis promoting effect. In further experiments by using differentiation media containing a defined composition of nutrients and growth factors, the influence of the medium on cardiomyogenesis and differentiation of CVPCs could be determined.

5.4 Influence of LIF on differentiation

LIF promotes cardiomyogenesis in EBs (Bader et al. 2001) and the addition of LIF isoforms to the medium of monolayers of CVPCs is a promising way to promote cardiomyogenesis in CVPCs. Although slight fluctuations in the outcome of CMC differentiation were present, as expected both M-LIF and D-LIF promoted cardiomyogenesis in experiments with clone H3 and clone B5. R-LIF the recombinant LIF-isoform isolated from bacteria and lacking posttranslational modifications did not have a clear effect.

Like in mESCs, LIF in CVPCs is an ambiguous factor inhibiting differentiation when CVPCs are cultivated on feeder cells and inducing differentiation when high concentrations of LIF are added for 4 days to sub-confluent monolayers of CVPCs growing on gelatin-coated culture plates. An explanation to this paradox is that excess LIF preferentially activates the ERK1 pathway whereas less LIF keeps CVPCs and ESC undifferentiated by activating the JAK/STAT-pathway. The MAPK-pathway and JAK/STAT-pathway are antagonizing each other (Niwa et al. 2009). Furthermore, undifferentiated CVPCs are almost confluent when grown on feeder cells in contrast to CVPCs seeded for differentiation thereby changing the concentration of LIF per CVPC. Another explanation is that LIF only promotes self renewal and proliferation of CVPCs and thereby increases cardiomyogenesis and that differentiation starts after the 4 day treatment with LIF. In further experiments Hoebaus (Hoebaus, 2009) showed that in CVPCs cultivation SNL76/7 feeder cells can be substituted by M-LIF without any loss of self-renewal capability.

5.5 CVPCs mRNA expression profile: Stem cell markers and cardiac marker gene expression

Stem cells have to express stemness markers to maintain their self-renewal capability. Recently a study showed, that 3 main transcription factors exist, which regulate self-renewal (Ying et al. 2008). Oct3/4, Nanog and Sox2 are the key stemness markers in mESCs. Although CVPCs are not embryonic stem cells they showed some self-renewal

capability by proliferating for at least 60 passages without loss of self-renewal and differentiation potential. Therefore, we were interested in the expression of Oct3/4, Nanog and Sox2 in CVPCs.

The Semi-quantitative RT-PCR showed that CVPCs transcribe all 3 essential transcription factors Oct3/4, Nanog and Sox2 which are required for stem cell self-renewal. This result confirms that CVPCs probably have stem cell character. Besides CVPCs self-renewal might be regulated similar to mESCs.

Another stemness marker is the enzyme telomerase. Telomerase consists of an RNA component (TER) that carries a short template complementary to telomere DNA, and a reverse transcriptase (TERT) that copies the RNA template to the 3'-end of linear DNA. The telomerase enzyme elongates telomeres of linear chromosomes in eukaryotes which otherwise would shorten during each cell division until cells become senescent or apoptotic. By inhibiting senescence telomerase thereby allows unlimited cell divisions. TERT is highly expressed in cells that divide extensively like some immune cells of eukaryotes, germ cells, stem cells and in cancer cells. Somatic cells express TERT at very low levels. RT-PCR showed that CVPCs do express mTERT. But in comparison to mESCs AB2.2 the expression level is low which is typical for adult stem cells and progenitor cells (Harrington 2004). In further experiments the enzymatic activity of telomerase in CVPCs should be determined and compared to that of mESCs. To determine the enzymatic activity a telomeric repeat amplification protocol (TRAP) assay has to be done. In TRAP cells are lysed and the telomerase is extracted. Afterwards a DNA substrate similar to telomeres is mixed with the telomerase and the quantity of elongated fragment is determined by PCR (Kim et al. 1994).

Besides expression of self-renewal transcription factors in CVPCs, cardiac-specific gene expression was of interest. Furthermore, the up-regulation of these genes during differentiation of CVPCs to CMCs was investigated. A comparative semiquantitative RT-PCR of undifferentiated CVPCs and CVPCs which had differentiated for 16 days was done. The RT-PCR showed that transcription factors Nkx2.5 and GATA4 which are expressed in differentiating CMCs are not present in undifferentiated CVPCs. But after 16 days of differentiation strong Nkx2.5 and GATA4 expression was detected. This expression of CMC transcription factors matches with the observation that CVPCs differentiate to beating CMCs within 16 days. MEF2C is a transcription factor which is necessary for correct development of heart and vasculature in embryos (Bi et al. 1999). MEF2C is present in CVPCs at day 0 and was upregulated during CVPCs differentiation in all clones, also in G3 which was confirmed in further experiments (Hoebaus, 2009). The myocyte specific proteins Tropomyosin and Desmin were not present in CVPCs at day 0 but up-regulated in CVPCs day 16. Isl1 is a LIM-homeodomain transcription factor which is expressed in cardiac progenitor cells of the second heart field during embryogenesis. Isl1 was found to be expressed in CVPCs at day 0 and at higher levels in CVPCs day 16. The RT-PCR showed that early premyocardial transcription factors like

MEF2C and Isl1 are expressed in CVPCs and are upregulated during differentiation. Muscle specific proteins like Tropomyosin and Desmin and early precardiac transcription factors like Nkx2.5 and GATA4 were present in differentiated CVPCs at day 16.

In summary, CVPCs express transcription factors OCT3/4, Sox2 and Nanog which maintain self-renewal in mESCs. They express telomerase at low levels in comparison to mESCs but its enzymatic activity needs to be determined. Early premyocardial transcription factors are expressed and myocardial specific transcription factors are upregulated during autonomously differentiation. Myocyte specific proteins are expressed in differentiated CVPCs. CVPCs mRNA expression shows stem cell and cardiac characteristics.

5.6 Translation of Nkx2.5 during CVPCs

The Nkx2.5 homeodomain protein is a marker of early precardiac cells and essential for heart formation in embryos (Durocher et al. 1997). Nkx2.5 is observed in heart tissue during the time of cardiac differentiation (Harvey et al. 2002). While RT-PCR showed that Nkx2.5 is upregulated in CVPCs between day 0 and day 16 of differentiation we were interested in the time at which Nkx2.5 is upregulated on a translational level. Therefore, monolayer differentiation experiments with CVPCs clone H3 were done and each 24 hours a well with CVPCs was harvested. The protein concentrations were normalized and prepared for SDS-PAGE. The SDS-PAGE was then western blotted with Nkx2.5 antibodies. Western blotting revealed that Nkx2.5 proteins are detectable for the first time after 48 hours of differentiation. The Nkx2.5 protein concentration continued to rise until day 6. At day 7 a reduction of Nkx2.5 was observed. Thus, the cardiac transcription factor Nkx2.5 was continuously up-regulated during differentiation of CVPCs on a protein level after 24 hours and rose between days 2 and 6. A decrease of Nkx2.5 began at day 7. For further information on Nkx2.5 translation levels further samples of differentiating CVPCs have to be taken and compared.

5.7 Influence of SPARC and BMP2 on differentiation

SPARC and BMP2 are versatile proteins which have different effects on different cell types. It was shown that SPARC is expressed during muscle development and in regeneration of muscle tissue and it is present in myoblasts, myotubes and muscle fibers. During embryogenesis SPARC is expressed in the heart primordial. It was shown that SPARC promotes cardiomyogenesis in EBS (Stary et al. 2005).

Without BMP2, early embryos have an abnormal cardiac development and fail in amnion and chorion formation (Zhang and Bradley 1996). BMP2 is essential for early heart development and induces expression of cardiac marker genes like Nkx2.5 and

GATA4 (Schlange et al. 2000). Stary (Stary et al. 2005) demonstrated a synergistically cardiomyogenesis promoting effect of SPARC and BMP2 in EBs. All this evidence led to the hypothesis that SPARC and BMP2 have a cardiomyogenesis promoting effect on CVPCs.

First of all, the mRNA expression of SPARC and BMP2 in CVPCs was determined. Semiquantitative RT-PCR showed that SPARC-mRNA is transcribed in CVPCs and in differentiating CVPCs at day 16. BMP2 was not expressed in undifferentiated CVPCs but at low levels in day 16 CVPCs. Nevertheless BMP2 could influence the differentiation of CVPCs because fetal bovine serum (FBS) contains several BMPs and a study in 2006 showed that BMPs can be purified from FBS (Kodaira et al. 2006). Thus, BMPs are present in the medium during CVPC differentiation. However, if BMPs in fetal bovine serum are specifically inhibited, myogenic differentiation is stimulated (Kodaira et al. 2006). But the monolayer CVPC differentiation experiments with different concentrations of FBS showed that a high FBS concentration and thus a high BMP concentration are favorable for cardiomyogenesis.

To affirm the SPARC expression on a translational level an immunofluorescence microscopy of differentiated CVPCs at day 16 was done. Immunostaining showed that each CVPC at day 16 is full of structures resembling vesicles containing SPARC. If the structures are vesicles, SPARC is probably secreted as an autocrine differentiation factor. Interestingly, all differentiated CVPCs contained SPARC regardless of cell type.

Secondly, the effect of adding an excess of SPARC to differentiating CVPCs was investigated. CVPCs were treated with 2 µg/ml for the first 4 days of differentiation and compared to untreated CVPCs. The addition of SPARC strongly promoted cardiomyogenesis. Notably, the onset of beating CMCs was at the same at day 16 but the number of beating CMCs was much higher in SPARC treated cells. On average, a 3-fold higher number of CMCs was counted in CVPCs treated with SPARC. The conclusion is that SPARC promotes cardiomyogenesis in vitro in monolayer differentiation. The same effect was demonstrated in embryoid bodies and during embryogenesis. Differentiation of CVPCs to CMCs is not accelerated by SPARC but more efficient.

Because M-LIF and SPARC had both shown a cardiomyogenesis promoting effect, a combination of them could improve cardiomyogenesis. Also because high FBS concentrations promoted cardiomyogenesis and FBS contains BMPs, the effect of excess BMP2 on cardiomyogenesis was investigated. CVPCs were treated with M-LIF for 4 days and afterwards SPARC, BMP2 and a combination of SPARC and BMP2 was added for 11 days. Again, M-LIF and SPARC treated CVPCs efficiently differentiated to CMCs. CVPCs treated with M-LIF only, also differentiated to CMCs but at a lower quantity. In comparison, M-LIF/SPARC treated CVPCs had a 2.5-fold higher average of active CMCs between day 12 and 19 than M-LIF treated CVPCs. CVPCs that were treated with M-LIF/BMP2 or M-LIF/BMP2 and SPARC did not differentiate to CVPCs.

Interestingly, BMP2 overruled the cardiomyogenesis promoting effect of both M-LIF or/and SPARC. In summary, SPARC enhances the cardiomyogenesis promoting effect of M-LIF. Surprisingly, BMP2 inhibited cardiomyogenesis at least at this concentration and in this experimental setup. However, further experiments showed that BMP2 promotes cardiomyogenesis when added for a shorter time period (Hoebaus, 2009). Because excess SPARC promotes cardiomyogenesis and SPARC is strongly expressed in CVPCs, the hypothesis was tested that an inhibition of endogenous SPARC with neutralizing antibodies inhibits cardiomyogenesis. In a new differentiation experiment CVPCs were treated with SPARC and SPARC antibodies and compared to CVPCs which were left untreated. As expected, SPARC promoted and SPARC neutralizing antibodies hampered cardiomyogenesis in CVPCs in comparison to untreated CVPCs. Treatment with SPARC antibodies resulted in less beating CMCs and CMCs started to beat 3 days later. Thus, endogenous SPARC is essential for the duration and the efficiency of cardiomyogenic differentiation of CVPCs.

5.8 SPARC signaling in CVPCs

To investigate if SPARC directly induces expression of mesodermal, precardiac and myocyte marker genes in CVPCs, CVPCs were treated with SPARC and the mRNA was isolated 0, 2, 4, 6 and 12 hours after treatment. As a control, CVPCs were treated with BMP2 to prevent differentiation. The expression of precardiac marker genes *Nkx2.5* and *GATA4*, the cardiac myocyte gene *desmin* and the cardiovascular precursor marker gene *Mesp1* were studied. Former RT-PCR experiments showed that *Nkx2.5*, *GATA4* and *Desmin* are expressed in differentiated CVPCs at day 16. But during the first 12 hours of SPARC treatment *Nkx2.5* was not expressed at detectable levels in SPARC or BMP2 treated CVPCs. *GATA4* was expressed at constant levels and in BMP2 treated CVPCs *GATA4* was down-regulated. The cardiovascular precursor marker gene *Mesp1* was down-regulated after SPARC and BMP2 treatment. The myocyte specific intermediary filament *desmin* was also not expressed yet. These results suggest that SPARC does not directly induce the expression of cardiomyogenesis specific factors.

To see when *Nkx2.5* expression is rising in differentiating CVPCs, an experiment with a larger time scale was designed. Besides the effects of M-LIF and BMP2 on *Nkx2.5* expression was investigated. CVPCs were treated with M-LIF, BMP2 and SPARC and combinations of M-LIF/BMP2 and M-LIF/SPARC and mRNA was isolated 24-, 48- and 72 hours after treatment. In SPARC and M-LIF treated CVPCs *Nkx2.5* was highly expressed after 48 hours. Whereas, in untreated and BMP2 or M-LIF/BMP2 treated cells *Nkx2.5* was up-regulated 24 hours later. The two experiments showed that SPARC does not directly up-regulate important pre-cardiac marker genes within 12 hours. In differentiating CVPCs both cardiomyogenesis promoting factors M-LIF and SPARC activated *Nkx2.5* transcription between 24 hours and 48 hours after addition, whereas

in untreated and BMP2 treated CVPCs Nkx2.5 transcription was apparent within 48 -96 hours.

However, how Nkx2.5 gets activated by SPARC or M-LIF remained unclear. Literature suggests that the Wnt pathway promotes Nkx2.5 expression and cardiomyogenesis in general. SPARC physically interacts with ILK1 (Barker et al. 2005) and ILK1 is known to inhibit GSK3 (Dedhar 2000) thereby activating the canonical Wnt pathway. Wnt is an essential regulator of cardiac progenitor cell differentiation (Cohen et al. 2008) and promotes differentiation of mesodermal progenitors into CMCs in EBs (Kwon et al. 2007). Furthermore, Wnt signaling promotes Nkx2.5 expression and early cardiomyogenesis in mouse embryonic carcinoma stem cells (Liu et al. 2009). Therefore, it is very likely that Wnt regulates differentiation in CVPCs as well. To test this hypothesis we wanted to inhibit GSK3 to activate the Wnt pathway and from the experiments done by Ying (Ying et al. 2008) the new specific GSK3 inhibitor CHIR99021 was known. But in these experiments with mESCs CHIR99021 was used to keep mESCs pluripotent and not to induce differentiation. As CVPCs partly resemble mESCs and share the same transcription factors which maintain self renewal the effect of GSK3 inhibition was unclear. In mESCs CHIR99021 and its inhibition of GSK3 was used to promote longevity and proliferation of mESCs. In our experiment we used the same concentration of CHIR99021 as in experiments with mESCs. In difference to prior experiments horse serum was used. This serum requires a higher concentration of CVPCs because CVPCs proliferate less. Horse serum is frequently used for myocyte differentiation and proved to be as effective as FBS for CVPC differentiation. Beating CMCs were observed from day 14 until day 26 in untreated CVPCs only. CVPCs treated with CHIR99021 remained undifferentiated with a small and round morphology. Almost no CMCs were observed in CHIR99021 treated CVPCs.

Thus, indirect activation of the Wnt pathway by inhibiting GSK3 maintains CVPCs undifferentiated. The experiment showed that total inhibition of GSK3 inhibits differentiation indicating that activating the Wnt pathway inhibits cardiomyogenesis in CVPCs. Therefore, SPARC does not exclusively indirectly activate Wnt during CVPCs differentiation. However, the differentiation-inhibitory effect of CHIR99021 may be a result of the long term treatment which could be circumvented by using CHIR99021 at different time points or by using other concentrations of CHIR99021. Further experiments with temporal Wnt activation and Wnt inhibition should be done with differentiating CVPCs.

6 Conclusion

All the results indicate that CVPCs, expressing stem cell markers, being able to self-renew and showing a limited potency which is restricted to the cardiovascular system, can be regarded as progenitor cells of the heart. The existence of CVPCs confirms the evidence that the heart is not a post-mitotic organ and that cardiomyocytes, vascular smooth muscle cells and endothelial cells can be replaced by stem cells in vivo (Anversa et al. 2006).

Although only cardiovascular cells were observed, all cells which develop during CVPCs differentiation should be identified by specific cellular markers. Furthermore, non-cardiovascular cell types should be excluded. The different cell types which develop should be quantified by FACS (fluorescence activated cell sorting). The transcriptional profile of CVPCs should be completed and expanded with real time PCR during differentiation. A 3-dimensional matrix similar to heart tissue could enhance the differentiation of CVPCs. An in vivo model for medical applications could be constructed by engrafting CVPCs into infarcted hearts.

SPARC is of special interest because it is expressed during heart formation in the embryo and in regeneration of muscle cells in vivo. In vitro SPARC enhanced the differentiation of CVPCs to CMCs. In vivo SPARC might induce regeneration of the heart as well. To be useful for medical applications all findings should be confirmed in human CVPCs.

7 References

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8 Appendix

8.1 Abstract

The treatment of cardiovascular disease with regenerative stem cell therapy is a promising new field of medical science. To achieve a targeted differentiation of stem cells to heart cells, it is mandatory to understand which factors are responsible and which signalling pathways are regulated. The ability to isolate, cultivate and handle cardiovascular progenitor cells would ease directed heart-specific differentiation.

By using a new method, cardiovascular progenitor cells (CVPCs) were isolated from heart tissue of neonatal mice and cultivated on feeder cells. CVPCs could be cultivated exactly like mouse embryonic stem cells (mESCs).

Like in mESCs, the property of self renewal and to remain undifferentiated was observed in CVPCs during continuous propagation over 60 passages. Stem cell characteristics were demonstrated by detection of expression stem cell specific transcription factors Oct3/4, Sox2 and Nanog by reverse transcriptase polymerase chain reaction (RT-PCR).

In vitro experiments in hanging drop culture, which were done exactly as in embryoid body experiments showed multipotency in CVPCs which is restricted to cardiovascular cells. Endodermal cells, smooth muscle cells and contracting cardiomyocytes were observed. Cardiac troponin T positive filaments were visualized by immunofluorescence. Differentiation experiments in common cell culture confirmed that CVPCs differentiate to cardiomyocytes autonomously. Mesodermal and heart specific markers were upregulated during differentiation.

Differentiation was always dependent on cell density, indicating autocrine regulation. Secreted protein acidic and rich in cysteine (SPARC) is a glycoprotein which is secreted by parietal endoderm during embryogenesis. RT-PCR revealed that SPARC is also expressed in CVPCs in their undifferentiated and differentiated state. Under the chosen specific conditions, administering SPARC and administering the cytokine leukemia inhibitory factor (LIF) promoted the specific differentiation of CVPCs to cardiomyocytes. The cytokine bone morphogenetic factor 2 (BMP2) which belongs to the transforming growth factor β (TGF- β) super-family inhibited cardiomyogenesis in CVPCs. Specific inhibition of glycogen synthase kinase 3 α and β (GSK3 α/β), a negative regulator of the Wnt-pathway prevented differentiation of CVPCs.

8.2 Zusammenfassung

Die Behandlung von kardiovaskulären Erkrankungen mittels regenerativer Stammzelltherapie ist eine vielversprechende, neue medizinische Heilungsmethode und Gegenstand zahlreicher Forschungsprojekte. Um eine gerichtete Differenzierung von Stammzellen zu Herzzellen zu ermöglichen, ist es dabei von großer Bedeutung herauszufinden, welche Faktoren dafür verantwortlich sind und welche Signalwege in der Zelle aktiv sind. Herz-Vorläuferzellen würden hierbei im Vergleich zu pluripotenten Stammzellen die gezielte Differenzierung zu Kardiomyocyten erheblich vereinfachen.

Mit einer neuen Methode wurden kardiovaskuläre Vorläuferzellen (CVPCs) aus dem Herzgewebe neonataler Mäuse isoliert und auf Feeder Zellen kultiviert. CVPCs konnten unter den gleichen Bedingungen wie embryonale Stammzellen der Maus (mESC) kultiviert werden. Wie bei mESC wurde eine hohe Fähigkeit zur Selbsterneuerung und zum Verbleib im undifferenzierten Zustand der CVPCs bei der kontinuierlichen Kultivierung über 60 Passagen festgestellt. Der Stammzellcharakter der CVPCs wurde auch durch den Nachweis der Expression der stammzellspezifischen Transkriptionsfaktoren Oct3/4, Sox2 und Nanog mittels Reverser-Transkriptase Polymerase Kettenreaktion (RT-PCR) demonstriert.

In vitro Experimente in Kultur mit hängenden Tropfen, die ähnlich zu Embryoid Body Experimenten mit mESC durchgeführt wurden und bei denen sich sogenannte Cardiac Bodies bildeten, zeigten, dass CVPCs im Gegensatz zu mESC eine limitierte Potentialität besitzen, die auf kardiovaskuläre Zelltypen beschränkt ist. Es bildeten sich endodermale Zellen, glatte Muskelzellen und schlagende Kardiomyocyten. Das herzspezifische Protein Cardiac Troponin T konnte in den Cardiac Bodies mittels Immunfluoreszenz sichtbar gemacht werden. Auch in normaler Zellkultur zeigte sich, dass CVPCs autonom zu schlagenden Kardiomyocyten differenzieren. Mesodermale und herzspezifische Marker wurden während der Differenzierung hochreguliert.

Die Differenzierung war dabei immer von der Zelldichte abhängig, was auf eine autokrine Regulierung der CVPCs hinwies. Secreted protein acidic and rich in cysteine (SPARC) ist ein Glykoprotein das während der Embryogenese vom parietalen Endoderm produziert wird. Wie RT-PCR Experimente zeigten, wird SPARC aber auch in CVPCs sowohl im undifferenzierten Zustand als auch in der differenzierten Zelle transkribiert. Zugabe von rekombinantem SPARC zu CVPC Kulturen verstärkte die spezifische Differenzierung der CVPCs zu Kardiomyocyten. Unter den gewählten Bedingungen wurde eine erhöhte Differenzierung der CVPCs zu Kardiomyocyten durch das Zytokin Leukemia Inhibitory Factor (LIF) ausgelöst. Bone morphogenetic factor 2 (BMP2), ein Zytokin der transforming growth factor beta (TGF β) Superfamilie, inhibierte die Kardiomyogenese in CVPCs. Spezifische Inhibierung der Glykogen

Synthase Kinase 3 α und β (GSK3) ein negativer Regulator des WNT Signalweges
verhinderte eine Differenzierung der CVPCs.

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